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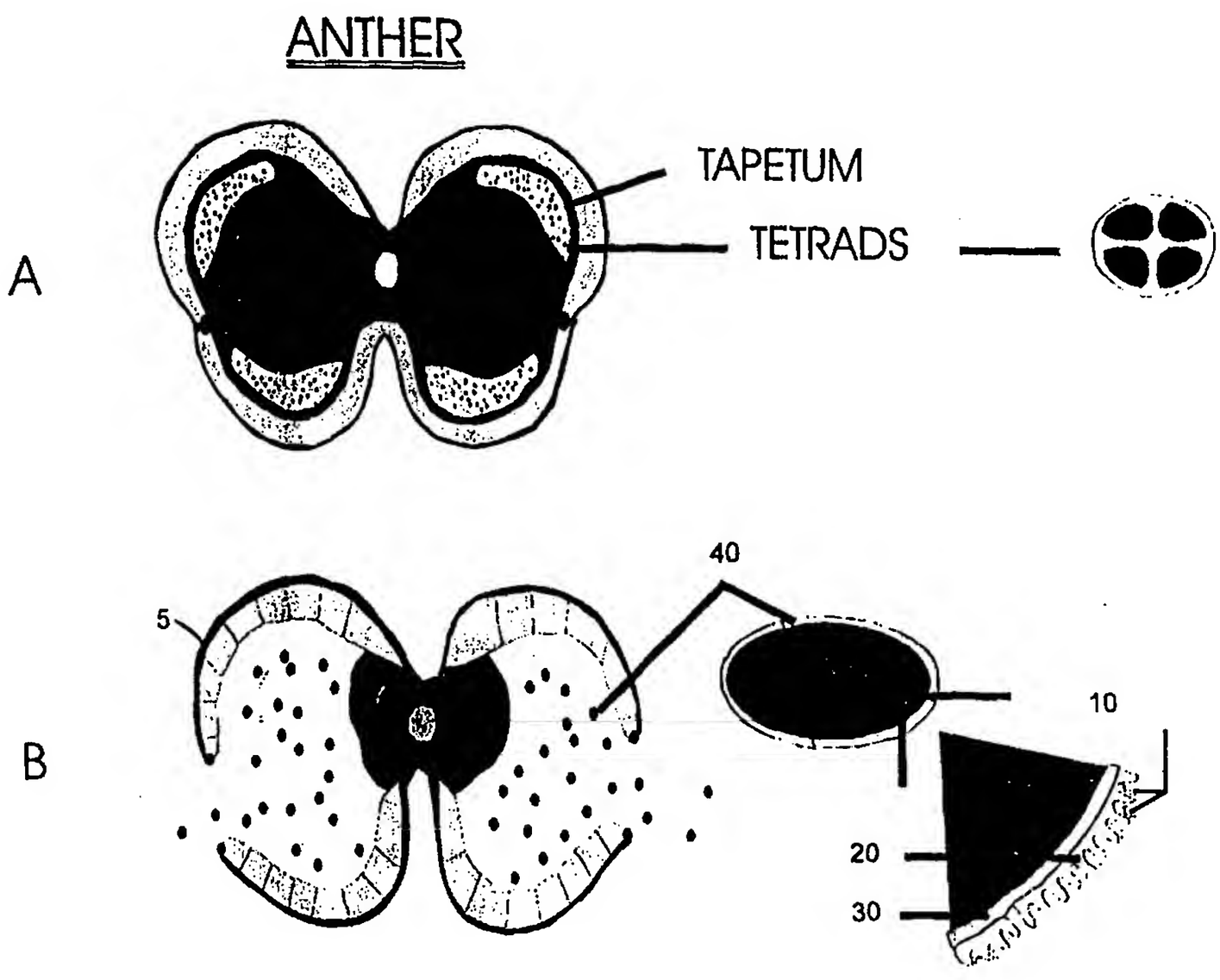
(54) Title: PROTEIN EXPRESSION IN FLORAL CELLS

(57) Abstract

This invention is directed to a method for the expression of a gene of interest, or a chimeric or modified gene allowing the localization of a protein, protein fusion, peptide or fragment of interest within the extracellular domain of a floral cell. This method comprises preparing a construct comprising a promoter sequence capable of expressing a gene encoding the protein, protein fusion, peptide, or fragment of interest, within the floral cell; a translated sequence of the protein, protein fusion, peptide, or fragment of interest, which is localized within the extracellular domain of a floral cell; a gene that encodes the protein, protein fusion, peptide, or fragment of interest; and a terminator sequence, and transforming a plant. Plants transformed with such a construct are characterized as having a protein, fragment thereof, or peptide of interest on the surface of a floral cell. Such localized proteins or peptides may be used for the purposes of peptide display, mediating plant sterility, modifying pollen–pistil interactions, altering pollen for insect consumption etc.



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PROTEIN EXPRESSION IN FLORAL CELLS

The present invention relates to the expression of proteins within plant tissues. More specifically, this invention relates to the expression of proteins in floral cells including those associated with anther and pistil.

BACKGROUND OF THE INVENTION

Pollen production is essential to the sexual reproductive success of the flowering plant. Male gametogenesis is a highly regulated developmental process which occurs within the diploid sporophytic tissue of the anther. It comprises three major phases: the differentiation of the sporogenous cells and meiosis; the development of the free uninucleate microspores; and the pollen maturation following microspore mitosis and ending with the formation of mature pollen (Scott, R., Hodge, R., Paul, W., Draper, J. *Plant Sci.* **80**:167-191 (1991)). Typically, pollen captured by a receptive stigma of the pistil will germinate and the pollen tube will grow extracellularly through the stigma and style until it reaches the ovule where it releases its nuclei that effect double fertilization. Similarly to a seed, the pollen accumulates reserves which enable it to germinate on a receptive stigma.

Normal pollen development is dependent upon the tapetum, a cellular layer lining the locular space of the anther. The tapetum provides the developing microspores with nutrients and other necessary products such as enzymes and structural components (Pacini, E., Franchi, G.G., Hesse, M. *Plant Syst. Evol.* **149**:155-185 (1985)). In *Brassica*, the secretory tapetum is made up of cells which are metabolically very active until about microspore mitosis at which time they degenerate (Grant, I., Beversdorf, W.D., Peterson, R.L. *Can. J. Bot.* **64**:1055-1068 (1986); Murgia, M., Charzynska, M., Rougier, M., Cresti, M. *Sex. Plant Reprod.* **4**:28-35 (1991); Polowick, P.L., Sawhney, V.K. *Sex. Plant Reprod.* **3**:263-276 (1990)). When the tapetal cells degenerate they release their cellular contents into the anther locule where they are thought to contribute to the formation of the external pollen coat (Evans,

D.E., Taylor, P.E., Singh, M.B., Knox, R.B. *Planta* **186**:343-354 (1992); Heslop-Harrison, J. *New Phytol.* **67**:779-786 (1968)). The pollen coat (sporoderm) consists of two layers, the exine (outer wall) and the intine (inner wall). The exine can be further subdivided into the nexine and sexine layers and is often elaborately sculptured and patterned (Scott, R.J. In: *Molecular and Cellular Aspects of Plant Reproduction* (eds) Scott, R.J., Stead, M.A. **55**:49-81 (1994)).

The interstices of the exine contain various substances including proteins, enzymes, lipids and allergens (Knox, R.B. In: *Embryology of Angiosperms*, (ed) Johri, B.M. pp. 197-271 (1994)) many of which are of tapetal origin. The lipidic and proteinaceous layer coating the exine is also called the tryphine. The mature pollen grain released upon anther dehiscence is dry and the drying process causes the tryphine to retract into the exine cavities. Numerous pollen enzymes have been identified (Brewbaker, J.L. In: *Pollen: Development and Physiology* (ed) Heslop-Harrison, J. pp. 156-170 (1971); Hiscock, S.J., Dewey, F.M., Coleman, J.O.D., Dickinson, H.G. *Planta* **193**:377-383 (1994); Knox, R.B. In: *Pollen: Development and Physiology* (ed) Heslop-Harrison, J. pp. 171-173 (1971); Lavithis, M., Bhalla, P.L. *Sex. Plant Reprod.* **8**:289-298 (1995); Travis, J., Whitworth, T., Matheson, N., Bagarozzi, D. *Acta Biochim. Pol.* **43**:411-418 (1996)). Many of these enzymes are located in the pollen coat especially in the intine layer and are readily elutable from the pollen grain. Some of these enzymes such as pectate lyases and ribonucleases have been shown to correspond to pollen allergens (Knox, R.B., Suphioglu, C. *Sex. Plant Reprod.* **9**:318-323 (1996)).

Recently, genes encoding some of the pollen coat proteins have been isolated. The *PCP*[7] gene encodes a pollen coat peptide from *Brassica oleracea* which has been shown to interact with *S*-locus glycoproteins (Doughty, J., Hedderson, F., McCubbin, A., Dickinson, H. *Proc. Natl. Acad. Sci. USA* **90**:467-471 (1993); Hiscock, S.J., Doughty, J., Willis, A.C., Dickinson, H.G. *Planta* **194**:367-374 (1995)). The *PCP1* gene encodes a cysteine-rich protein which may be involved in pollen-stigma interactions in *Brassica oleracea* and which belongs to a family of 30 to 40 genes

(Stanchev, B.S., Doughty, J., Scutt, C.P., Dickinson, H., Croy, R.R.D. *Plant J.* **10**:303-313 (1996)). This gene was shown to be expressed gametophytically and its product is released from the pollen protoplast into the surface coating.

There have also been numerous genes isolated which show expression in the tapetum, yet the function of the proteins they encode (Schrauwen, J.A.M. *Acta Bot. Neerl.* **45**:1-15 (1996)), and whether they associate with the pollen coat is largely unknown. Genes encoding β-1,3-glucanase have been shown to be expressed in the tapetum and these enzymes are involved in breaking down the callose wall surrounding the tetrads releasing the microspores (Bucciaglia, P.A., Smith, A.G. *Plant Mol. Biol.* **24**:903-914 (1994); Hird, D.L., Worrall, D., Hodge, R., Smartt, S., Paul, W., Scott, R. *Plant J.* **4**:1023-1033 (1993)). There are only two different examples of tapetal-specific genes (ie. expressed sporophytically) whose products were shown to be localized to the pollen coat. The related genes *Sa*tap35 and *Sa*tap44 from *Sinapis alba* are associated with the exine of the developing microspore and may be involved in sporopollenin formation and/or deposition (Staiger, D., Kappeler, S., Müller, M., Apel, K. *Planta* **192**:221-231 (1994)). The *Sta* 41-2 and *Sta* 41-9 genes from *Brassica napus* encode proteins which possess a hydrophobic domain similar to that of the seed oleosins (Robert, L.S., Gerster, J.L., Allard, S., Cass, L., Simmonds, J. *Plant J.* **6**:927-933 (1994)). Sequence similarity among the *Sta* 41-2 and *Sta* 41-9 genes and seed oleosin genes from *Brassica napus* (Murphy, D.J., *Prog. Lipid Res.* **32**:247-280 (1993)) are limited to the relatively small hydrophobic domain and show levels of 30-36% identity. These tapetal expressed genes have now been demonstrated to belong a large family of related anther oleosin-like genes in *Brassica* (Ross, J.H.E., Murphy, D.J. *Plant J.* **9**:625-637 (1996); Ruiter, R.K., Van Eldik, G.J., Van Herpen, R.M.A., Schrauwen J.A.M., Wullems, G.J. *Plant Cell* **9**:1621-1631 (1997)). Unlike the *Sa*tap35 and *Sa*tap44 proteins, the oleosin-like proteins do not possess a signal peptide and are thought to be released passively into the anther locule upon tapetum degeneration by association with lipids released from the tapetum or found as part of the tryphine of the pollen coat. Without wishing to be bound by theory, the hydrophobic region of the tapetal oleosin-like protein may be required for localization

upon the pollen coat by association with lipids. The tapetal oleosin-like proteins constitute the major protein of the *Brassica* pollen tryphine and they occur as post-translationally cleaved protein products (Ross, J.H.E., Murphy, D.J. *Plant J.* **9**:625-637 (1996)). The function of the tapetal oleosin-like proteins is unknown but they may play a role in the interaction between the pollen and the stigma the specialized part of the pistil which receives the pollen.

The stigma is responsible for capturing and selecting compatible pollen grains and for facilitating their germination. Angiosperm stigmas have been classified morphologically as 'dry' stigmas having an extracuticular proteinaceous pellicle but no free-flowing secretion or 'wet' stigmas which are covered by a secretion at the receptive stage (Heslop-Harrison, Y., Shivanna, K.R. *Ann. Bot.* **41**:1233-1258 (1977)). In *Brassica*, the dry stigma is the site of the sporophytic self-incompatibility (SI) response with incompatible pollen being unable to grow through the stigmatic papillar cells or failing to germinate altogether.

A number of genes have been shown to be preferentially expressed in the *Brassica* stigma and most of these genes correspond to genes associated with SI: *SLG* (*S*-locus glycoprotein), *SRK* (*S* receptor kinase; U.S. 5,484,905) or *SLR* (*S*-locus-related; WO94/25613) genes (for review: Nasrallah, J.B., Nasrallah, M.E. *Plant Cell* **5**:1325-1335 (1993)). The products of the *SLG* and *SRK* genes are believed to be involved in a signal pathway modulating the SI reaction in response to a ligand carried by the pollen grain. WO94/25613 is directed to pistil-, and anther-specific gene expression. It discloses the cloning of several SLG's genes and the isolation of the SLG_1 promoter region, and the preparation of transcriptional fusion products using the promoters from the SLG genes. Furthermore, U.S. 5,585,543 discloses several genes related to the S-locus.

Another example of a gene highly expressed in the *Brassca* stigma is Pis 63 (Robert, L.S. et al Plant Mol. Biol 26: 1217-1222 (1994)). The promoter obtained from the genomic clone GPIS 363, which contains gene *Pis* 63-2 was shown to direct

the expression of the marker gene β-glucuronidase transiently in *B. napus* stigmas and stably in the stigmas of transformed tobacco plants (Robert et al, Plant Cell Rep. 18: 357-362 (1999)).

The SI response in *Brassica* provides an example that a molecular based interaction between the pollen grain and the stigmatic papillae exists and that such an interaction can be modified or mimicked by targeting polypeptides to the appropriate part of the pollen and/or stigma. It is thought that localization of the SLG proteins arrises as a result of the appropriate signal peptide directing the protein extracellularly, following expression.

The preparation of plants with female sterility based on a style-stigma specific "STMG"gene and derived constructs using PSTMG promoter cassettes is disclosed in U.S. 5,633,441. These constructs include transcriptional fusions comprising barnase, papain or RNAse. In U.S. 5,652,354, the use of stamen-selective promoters useful in driving expression in anther, pollen, or filament cells, especially in the tapetum or anther epidermal cells is disclosed. U.S. 5,571,904 is directed to male flower specific gene sequences. Genomic clones of pMS10, 14 and 18 were obtained and promoter cassettes were constructed using MS10. There is also evidence presented where pMS14 expression has been localized within the tapetal cell layer. Other publications also disclose floral-specific gene and associated regulatory elements. For example, U.S. 5,633,438 discloses microspore-specific regulatory element, Bnm1; U.S. 5,545,546 discloses the cloning of W2247, a pollen specific promoter obtained from maize (inbred corn line W22); U.S. 5,659,124 teaches use of existing anther specific promoters to produce male sterile plants; WO92/13957 is directed to the cloning of CA444 which is a stamen/anther specific gene; WO97/13401 discloses the cloning of a rice tapetal specific gene RTS2; WO93/25695, is directed to the preparation of male sterile plants using tapetal specific promoters such as those from the TA29 gene or PT72; CA 2,099,482, teaches the disruption of the formation of viable pollen resulting in male sterile plants using an anther specific promoter; CA2,106,718 is directed to the disruption of normal pollen development using anther specific promoters driving

chimeric constructs that disrupt pollen development; Worrall D, et al (Plant Cell 4:759-771 (1992)) teaches the use of a tapetal specific promoter to drive the expression of callase which prematurely degrades the callose wall surrounding the developing tetrad of microspores thereby releasing the microspores into the anther locule. This premature release of microspores leads to male sterility. However, there is no teaching of modifying the extracellular domain of a free microspore or pollen grain; CA2,165,934 discloses the use of a polygalacturonase promoter to drive a chimeric construct within microspores of *Brassica napus* plants.

Based upon the review of the prior art, there are two known mechanisms that exist for the targeting of a protein onto the pollen coat, either by deposition following tapetal degradation, or as a result of extracellular targeting either from the tapetal cells or microspore cells via a signal peptide. Similarly, extracellular targeting of pistil-derived gene products, for example the SLG gene product, appears to involve the use of a signal peptide. However, none of the prior art publications disclose modifying the protein composition of the microspore/pollen coat or the interactions of these proteins with the stigma or pistil. Rather, most of the published literature is directed to producing sterile plants through the disruption of pollen development. Although, disruption does not occur by modifying the extracellular compartment. Nor does the prior art teach a similar modification of the stigma cells using chimeric gene constructs that would affect the interaction between these cells and pollen while all of these cell types could remain viable. The approach described herein is primarily directed at modifying pollen or stigma function, and in some instances affects the interaction between pollen and stigma.

There is no teaching of the preparation of transcriptional or translational fusion proteins specifically designed to localize on the exterior of a pollen or stigma cell. For example, but not limited to, comprising hydrophobic domains of pollen coat proteins and the like, to direct the translocation of the fusion product to the exterior surface of the pollen. Furthermore, beyond uses that are directed to pollen disruption for the production of sterile plants, the prior art does not disclose methods that provide for

peptide display, antibody production, altering the food value of pollen for human consumption, the use of treating insects, or alleviating allergenic responses by specifically targeting protein products to the surface of the appropriate floral cell.

This invention relates to a method of modifying the extracellular compartment of floral cells, including targeting proteins, protein fusions, or peptides to this extracellular domain. Methods using chimeric gene constructs that allow targeting of proteins, fusion proteins or peptides of interest to cells of the pistil, microspore, or pollen coat are disclosed and exemplified.

SUMMARY OF THE INVENTION

The present invention relates to the expression of proteins within plant tissues. More specifically, this invention relates to the expression of proteins within the extracellular compartment of floral cells including those associated with anther and pistil.

According to the present invention there is provided a method (A) for modifying the extracellular compartment of a floral cell of a plant, the method comprising, expressing a construct comprising a gene of interest within an anther or stigma cell, the gene of interest encoding a protein, fusion protein or peptide, or a fragment of said protein, fusion protein or peptide, the protein, fusion protein or peptide, or a fragment of the protein, fusion protein or peptide capable of modifying the composition of the extracellular compartment of the floral cell and altering either the function, use or development of the floral cell, or modifying the interaction of the floral cell with other cells. This invention relates to the above method wherein the gene of interest is native, or non-native, to the plant, or wherein the construct is a chimeric gene construct.

This invention relates to the method (A) as defined above wherein the floral cell is a pollen grain, and the protein, fusion protein or peptide, or a fragment of the protein, fusion protein or peptide is released into a locule of an anther thereby associating with the extracellular compartment of the pollen grain. This invention also embraces the above method, wherein the floral cell is either a pollen grain or a stigma cell, and the construct comprises a translated sequence capable of directing the extracellular localization of said protein, fusion protein or peptide, or a fragment of the protein, fusion protein or peptide on the floral cell. Preferably the translated sequence is selected from the group consisting of a signal peptide, a hydrophobic domain, or a combination thereof, or the translated sequence is a protein, or fragment thereof, known to be targeted to the extracellular compartment of a floral cell.

According to the present invention there is provided a method (B) for obtaining the localization of a protein, fusion protein, or peptide of interest, or a fragment of the protein, fusion protein, or peptide, within the extracellular compartment of a floral cell, comprising:

i) preparing a gene construct comprising:

a) a promoter sequence capable of expressing a gene encoding the protein, fusion protein, or peptide of interest, or a fragment of the protein, fusion protein, or peptide, within the floral cell;

b) a gene that encodes the protein, fusion protein, or peptide of interest, or a fragment of the protein, fusion protein, or peptide; and

c) a translated sequence capable of directing the extracellular localization of the protein, fusion protein, or peptide of interest, or a fragment of the protein, fusion protein, or peptide, on the floral cell;

d) a terminator sequence; and

ii) transforming a plant with the gene construct;

wherein the floral cell, is selected from the group consisting of pollen, anther or pistil cells.

Furthermore, this invention includes the method (B) as described above, wherein the translated sequence of step c) is selected from the group consisting of a signal peptide, a hydrophobic domain, or a combination thereof.

This invention is also directed to a method (C) of chemically linking a protein or peptide of interest to the pollen coat comprising:

a) activating pollen grains with a desired reagent for conjugation;

b) adding the protein of interest

This invention embraces a pollen grain prepared by the method (C) as described above.

Furthermore, this invention includes a microspore or pollen, or a combination thereof, prepared using the method (B) as described above. This invention is also directed to a transgenic plant cell, a transgenic plant comprising the microspore or pollen, or combination thereof prepared using the method (B) as described above, and to seeds obtained from the transgenic plant.

This invention also embraces a method (D) of modifying pollen-pistil interaction or function comprising, producing a microspore, pollen, or pistil cell, or combination thereof, within a plant using the method (B) as described above, so that the microspore, pollen, or pistil, or combination thereof comprise an extracellular protein, fusion protein, or peptide of interest, or a fragment of the protein, fusion protein, or peptide, that modifies pollen and pistil interaction or function. This invention also embraces a method (D), wherein the extracellular protein, fusion protein, or peptide of interest, or a fragment of the protein, fusion protein, is localized to the microspore, or pollen, or to the pistil cell, or combination thereof.

This invention also provides for a method (D) wherein the pollen-pistil interaction or function produces, mediates, or prevents self compatibility, self incompatibility out-crossing, in-crossing or a combination thereof.

This invention also relates to the method (D) as described above, wherein the protein, fusion protein, or peptide of interest, or a fragment of the protein, fusion protein, or peptide, is selected from the group consisting of protease, glucosidase, glycanase, nuclease, lipase, hydrolyase, toxin and antibody, or an active portion thereof.

This invention also embraces a vector comprising:

a) a promoter sequence capable of expressing a gene encoding a protein, fusion protein, or peptide of interest, or a fragment of the protein, fusion protein, or peptide, within a floral cell;

b) a gene that encodes the protein, fusion protein, or peptide of interest, or a fragment of the protein, fusion protein, or peptide;

c) a translated sequence capable of directing the extracellular localization of the protein, fusion protein, or peptide of interest, or a fragment of the protein, fusion protein, or peptide, on the floral cell; and

d) a terminator sequence,

and to a transgenic plant cell, transgenic plant, or seed obtained from the transgenic plant, comprising the vector defined above.

This invention also includes a pistil cell prepared using the method (B) as described above. Furthermore, this invention embraces a transgenic plant comprising a pistil cell prepared using the method (B) as described above. Also included are seeds obtained from this transgenic plant.

Furthermore, this invention relates to a pistil cell characterized in that the extracellular compartment of the pistil cell comprises a protein, fusion protein, or peptide of interest, or a fragment of the protein, fusion protein, or peptide encoded by a chimeric gene construct. Furthermore, this invention embraces a transgenic plant comprising a pistil cell that comprises a protein, fusion protein, or peptide of interest, or a fragment of the protein, fusion protein, or peptide encoded by a chimeric gene construct, or seed obtained from this transgenic plant.

This invention also embraces the method (B) as described above, wherein the protein, fusion protein, or peptide of interest, or a fragment of the protein, fusion protein, or peptide, is localized on the surface of a pollen grain for the purpose of peptide display, or it is an antibody or antigen, or it is effective in controlling insect growth, behaviour, feeding, development, or reproduction, or a combination thereof.

This invention is directed to modifying the protein composition of the extracellular domain of a microspore, or pollen coat, or the interactions of these proteins with the stigma, pistil, or other cells of interest, while possibly maintaining

the pollen, and the cells of the stigma, in a viable state. Furthermore, this invention relates to modifying the protein composition of the extracellular domain of stigma cells in order to affect the interaction between these cells and either unaltered or modified pollen grains, wherein each of these cell types could remain in a viable state. The prior art is directed to producing sterile plants through the disruption of pollen development. However, this disruption does not occur by modifying the extracellular domain of the pollen. The approach described herein is primarily directed at modifying pollen or stigma cell function, and in some instances affects the interaction between pollen and stigma. However, the methods disclosed within this invention are not necessarily disruptive to pollen development as is the case within the prior art, nor are they necessarily disruptive to pistil development.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features of the invention will become more apparent from the following description in which reference is made to the appended drawings wherein:

FIGURE 1 shows a drawing of anthers at two different stages of flower development and illustrates early and late pollen development. Figure 1A shows early flower development with an intact tapetal layer and tetrad of microspores. Figure 1B shows late flower development, with mature pollen grains having pollen coats (tryphine) containing tapetal debris.

FIGURE 2 shows a drawing of a pollen grain. Details of the components of the pollen coat are illustrated and an example of polypeptides targeted to the pollen coat is indicated.

FIGURE 3 is a schematic representation of three different translational fusions possible with the *Brassica napus* tapetal oleosin-like Sta 41-9 protein. Figure 3(A) shows a C-terminal translational fusion with a full length tapetal oleosin. Figure 3(B) shows a C-terminal translational fusion at a proteolytic cleavage

site. Figure 3(C) shows an internal translational fusion at a proteolytic cleavage site. The fused protein is indicated by a hatched box.

FIGURE 4 is a schematic representation of the construction of the *Brassica napus* tapetal oleosin-like *Sta* 41-9 translational fusion cassette OFC-1. Figure 4(A) shows the removal of the *Nde* I site in the promoter of *Sta* 41G(10) and the preparation of BH-1, NB-6 and KB-1 from mp 101. Figure 4(B) shows the preparation of SS-4 and OFC-1 from KB-1.

FIGURE 5 is a schematic representation of the construction of plant transformation vector TOG-1, the *Brassica napus* tapetal oleosin-like *Sta* 41-9/*E. coli* β-glucuronidase translational fusion.

FIGURE 6 is a schematic representation of the construction of the plant transformation vector TOP-1, the *Brassica napus* tapetal oleosin-like *Sta* 41-9/*Sitophilus zeamais* protease translational fusion.

FIGURE 7 is a schematic representation of the construction of the plant transformation vector TOPI-1, the *Brassica napus* tapetal oleosin-like *Sta* 41-9/*Onchocerca volvulus* protease inhibitor translational fusion.

FIGURE 8 is schematic representation of the construction of plant transformation vectors SPF-1 containing the *Brassica napus* GPIS363/*Sitophilus zeamais* protease translational fusion and SPIF-1 containing the *Brassica napus* GPIS363/*Onchocerca volvulus* protease inhibitor translational fusion.

FIGURE 9 is a schematic representation of the construction of plant transformation vector SPOV-1 containing an enhanced CaMV35S promoter directing the expression of the *Brassica napus* SLG_{WS1} signal peptide/*Onchocerca volvulus* protease inhibitor translational fusion.

FIGURE 10 is a schematic representation of the construction of plant transformation vector POV-1 containing the *Brassica napus* Sta44G(2)/*Onchocerca volvulus* protease inhibitor translational fusion.

FIGURE 11 is a schematic representation illustrating an example of the pollen, stigma and their interaction in a self compatible plant expressing TOP, a tapetal oleosin-like/protease fusion.

FIGURE 12 is a schematic representation illustrating an example of the pollen, stigma and their interaction in a self compatible plant expressing SPIF, a stigma protein/protease inhibitor fusion.

FIGURE 13 is a schematic representation illustrating an example of the pollen, stigma and their interaction in a hybrid self compatible plant containing TOP, a tapetal oleosin-like/protease fusion and SPIF, a stigma protein/protease inhibitor fusion.

FIGURE 14 is a schematic representation illustrating an example of the pollen, stigma and their interaction in a self compatible plant expressing SPF, a stigma protein/protease fusion.

FIGURE 15 is a schematic representation illustrating an example of the pollen, stigma and their interaction in a self compatible plant expressing TOPI, a tapetal oleosin-like/protease inhibitor fusion.

FIGURE 16 is a schematic representation illustrating an example of the pollen, stigma and their interaction in a hybrid self compatible plant containing TOPI, a tapetal oleosin-like/protease inhibitor fusion and SPF, a stigma protein/protease fusion.

FIGURE 17 is a schematic representation illustrating an example of the pollen, stigma and their interaction in a self incompatible plant expressing TOGL, a tapetal oleosin-like/glycanase fusion.

FIGURE 18 is a schematic representation illustrating an example of the pollen, stigma and their interaction in a self incompatible plant expressing SGL, a stigma protein/glycanase fusion.

FIGURE 19 shows *in vitro* germination of tobacco pollen after 3 h incubation in either casein, Figure 19 (A), papain Figure 19 (B) or cycloheximide Figure 19 (C).

FIGURE 20 shows the detection of the *Brassica napus* tapetal oleosin-like *Sta* 41-9/*Onchocerca volvulus* protease inhibitor fusion protein in transgenic *Brassica carinata* containing TOPI-1 (SEQ ID NO: 5). Figure 20 (A) Western blot analysis of anther protein extracts from 4 mm flower buds of different transgenic lines separated by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and cross-reacted with anti-CPI (cysteine protease inhibitor, unpublished results from Gleddie et al.) antibody. Lanes 1 to 13 represent 35 µg of proteins from transgenic lines No. 1 to 5, 7 to 12, 14 and 15; lane 14 contains 35 µg of proteins from an untransformed *B. carinata* plant and lane 15 contains 30 ng of OV7-GST (cysteine protease inhibitor OV7 conjugated to GST). The OV7/GST fusion protein has a predicted molecular weight of 42 kDa. The anti-CPI antibody cross-reacted with two bands in most transgenic plants. The higher molecular weight band corresponds to the full length fusion protein with a predicted molecular weight of 57 kDa, whereas the lower band has a molecular weight of approximately 47 kDa, which is the expected size of the processed fusion protein where the N-terminal end of the oleosin-like protein has been cleaved off. Figure 20 (B) Coomassie blue-stained SDS-PAGE of anther extracts from 4 mm flower buds. The sample lanes correspond to those described in Figure 20 (A). The cross-reacting bands are not evident following Coomassie blue staining.

FIGURE 21 shows the detection of the *Brassica napus* tapetal oleosin-like *Sta* 41-9/*Onchocerca volvulus* protease inhibitor fusion protein during the flower development of a transgenic *Brassica carinata* plant containing TOPI-1 (SEQ ID

NO: 5). Figure 21 (A) Western blot analysis of anther protein extracts from developing flower buds of transgenic line No. 1 separated by SDS-PAGE and cross-reacted with anti-CPI (cysteine protease inhibitor) antibody. Lanes 1 to 6 represent 30 μg of anther proteins from 2 mm, 3 mm, 4 mm, 5 mm, 6 mm and 7 mm flower buds respectively, lane 7 contains 30 μg of anther proteins from an untransformed *B. carinata* plant and lane 8 contains 30 ng of OV7-GST (predicted molecular weight of 42 kDa). The full length 57 kDa fusion protein was detected in anther protein extracts from 3 mm buds and was undetectable in the older 7 mm buds. However, the 47 kDa corresponding to the cleaved protein was evident in the anther protein extracts from 4 mm buds anthers and persisted through to the late stages of bud development. Figure 21 (B) Coomassie blue-stained SDS-PAGE of anther extracts from developing flower buds from transgenic *B. carinata* line No. 1 containing TOPI-1. The sample lanes correspond to those described in Figure 21 (A). The cross-reacting bands are not evident following Coomassie blue staining.

FIGURE 22 is a schematic representation of the construction of plant transformation vector SAS-1 containing the *Brassica napus* tapetal oleosin-like *Sta* 41 G(10) promoter fragment transcriptionally fused to the *B. napus* tapetal oleosin-like *Sta* 41-2 cDNA in the antisense orientation.

DESCRIPTION OF PREFERRED EMBODIMENT

The present invention relates to the expression of proteins within plant tissues. More specifically, this invention relates to the expression and localization of proteins within the extracellular compartment of floral cells including those associated with pollen and pistil.

Methods and compositions are provided for the targeting of proteins or peptides to the extracellular domain of a microspore, or pollen or pistil cells. The methods include preparing chimeric DNA constructs encoding a polypeptide, or a fusion polypeptide consisting of a microspore, pollen coat or pistil protein and a coding sequence for a polypeptide of interest. Inserting this DNA construct in a plant genome, and regenerating transgenic plants which produce pollen, stigmas, or both, with the polypeptide or fusion polypeptide.

As used herein "pollen function" includes processes associated with development of pollen, dispersal of the pollen, recognition, interaction and adhesion of the pollen to the stigma cells, pollen tube germination and pollen tube growth, and fertilization.

By "pistil function" it is meant processes associated with development of the pistil, interactions with pollen, including pollen capture, permitting or preventing pollen germination, pollen tube growth, fertilization, or a combination thereof, and nurturing zygote development.

By "extracellular compartment" or "extracellular domain" it is meant the region of the cell that includes, and lies outside, the plasmalemma. However, the extracellular compartment, or domain, may be associated with the cell in some manner. This compartment may comprise proteins that are anchored within the plasmalemma and that are displayed toward the outside of the cell, or proteins that are localized, via excretion or deposition, within the apoplast, cell wall or outer regions of the cell wall

such as the surface of the cell, or that are released within the locule. For example, in the case of pollen (40; see Figures 1 and 2), this compartment includes the anther locule (5), tryphine (10), and the pollen coat exine (20) comprising the nexine and sexine, and intine (30), as well as the pollen tube and compounds that are synthesised and excreted from within the pollen or pollen tube, or compounds that are deposited onto the outer wall during development of pollen, the pollen tube or locule. In the case of the pistil, this compartment includes the ovary and the style, including the transmitting tract and compounds that are synthesised and excreted from cells of this tract, or compounds that are deposited onto the outer cell walls during development of the tract. In the case of the stigma, this compartment includes the cuticle, and compounds that are synthesised and excreted outside the cuticle, such as compounds that are deposited onto the outer surface of the papillar cells including, but not limited to, the proteinaceous pellicle of *Brassica* stigmas, or secretions of tobacco stigmas.

By "directing extracellular localization" it is meant using a chimeric gene construct comprising motifs capable of targeting a protein or protein fusion or peptide of interest passively or actively to the extracellular compartment. For example, which is not to be considered limiting in any manner, such motifs responsible for actively directing extracellular localization may include sequences encoding signal peptides, or hydrophobic domains, for example fragments obtained from the tapetal oleosin-like protein, or a hydrophobic domain obtained from a seed oleosin or tapetal oleosin-like protein. Motifs responsible for passively targeting extracellular localization upon tapetal degradation may include, but are not limited to, protein primary structure or protein modifications affecting affinity to the extracellular domain. This localization may also comprise a transient association between the protein, fusion protein, or peptide of interest and the extracellular domain, such as enzyme substrate interactions, for example glycosidase-carbohydrate or protease-protein reactions.

By "gene of interest" it is meant a sequence nucleic acids that encode a protein. The gene of interest may be of native origin, in that it is obtained from the same species of plant within which it is to be reintroduced, or it may be of non-native origin,

i.e. it is obtained from a plant that is different from the plant to which it is to be introduced, or it is obtained from another source, i.e. bacterial, viral, animal etc. A gene of interest may comprise regulatory regions such as promoters, enhancers, terminator sequences and the like that are endogenous to the gene of interest with which they are isolated. However, a gene of interest may also be introduce within a vector along with other sequences, typically heterologous, to produce a chimeric construct.

By "chimeric DNA construct" or "chimeric construct" it is meant a nucleic acid molecule comprising regions of DNA sequences not normally associated with the gene of interest. These regions may be homologous or heterologous with respect to the gene of interest, and may be obtained from native or non-native sources. For example, a chimeric construct that results in a translational fusion product may include a native or heterologous enhancer region, a native or heterologous promoter region, followed by regions comprising a portion of a native or heterologous 5' coding region including such motifs as signal peptides, or hydrophobic domains as required, a native or heterologous DNA sequence capable of encoding a protein or peptide of interest, followed by 3' motifs that may also be involved in extracellular targeting or regulatory functions, or both, and a terminator region. It is to be understood that a range of 5' or 3' regions of the chimeric construct may be used in order to optimize synthesis of the final gene construct, expression of the gene product, and localization of the gene product within the extracellular compartment. Furthermore, a chimeric construct that results in a transcriptional fusion product may comprise a native or non-native enhancer and promoter region operationally fused with an optional signal peptide and the protein or peptide of interest, followed by a 3' regulatory, or terminator region, or a region comprising both a regulatory and terminating function as defined above.

By "modified gene" it is meant a gene whose sequence has been altered using methods known in the art such as but not limited to site-directed, or random mutagenesis, deletions, rearrangements, or fusions and the like.

By "fusion protein" it is meant proteins synthesized from chimeric DNA constructs. These proteins may comprise a portion of a native protein along with a heterologous protein comprising the protein of interest. Such a fusion protein may comprise a signal peptide, or hydrophobic domain, or other motif that permits targeting of the protein of interest to the extracellular compartment, for example, but not limited to, motifs obtained from an oleosin-like protein, or Sta 41-2 or Sta 41-9, Sta 44, SLG_{WS1} or GPis363..

By "expression cassette" it is meant a chimeric DNA molecule that includes transcriptional and translational regulatory sequences of DNA capable of expressing a chimeric gene whose product is subsequently targeted to the extracellular compartment of a floral cell. For example, an expression cassette may comprise promoter and regulatory sequences controlling the expression of genes, and the targeting of the encoded products within the tapetum or the pollen. However, this is not to be considered limiting in any manner as other constructs may also be directed to other extracellular compartments as previously defined. In the case of tapetal expression, the gene product may be expressed in the tapetum and subsequently translocated to the pollen or developing microspores, for example callase or oleosin-like proteins, or the protein may be expressed within the pollen and re-located to the microspore or pollen coat during development or germination, for example, pectate lyase or PCP1.

By "promoter" or "regulatory region", it is meant a region typically within a genomic sequence that has the property of controlling the expression of a DNA sequence that is operably linked with the regulatory region. Such regulatory regions may include promoter or enhancer regions, and other regulatory elements recognized by one of skill in the art. Typically this region comprises nucleotide sequences at the 5' end of a coding region, or fragment thereof that contain all the signals essential for the initiation of transcription and for the regulation of the rate of transcription. The promoters used to exemplify the present invention may be selected to ensure expression of a desired gene within the tissue of interest, or during appropriate stages of

development, for example, but not limited to, tapetal- (e.g.Sta 41; Hong et al. (1997) Plant Mol. Biol 34:549-555), pollen- (e.g. Sta 44; Hong et al. (1997) Plant Cell Rep. 16: 373-378)) or pistil- (e.g. Pis 63; Robert et al. (1999) Plant Cell Rep. 18: 357-362) specific promoters, and these and other promoters are, or would be, known to those of skill in the art. However, constitutive promoters may also be used such as, but not limited to, the CaMV 35S (Timmermans et al. (1990) J. Biotechnol. 14: 333-344), ubiquitin (Holton et al. (1995) Plant Molec. Biol. 29: 637-646) or action (An et al. (1996) Plant J. 10:107-121) promoters. Also included are inducible promoters which may also be used in order to regulate the expression of the gene following the induction of expression by providing the appropriate stimulus for inducing expression. The inducer can be a chemical agent such as a protein, metabolite, growth regulator, herbicide or phenolic compound or a physiological stress imposed directly by heat, cold, salt, or toxic elements or indirectly through the action of a pathogen or disease agent such as a virus. A plant cell containing an inducible promoter may be exposed to an inducer by externally applying the inducer to the cell or plant such as by spraying, watering, heating or similar methods.

The chimeric gene constructs of the present invention can further comprise a 3' untranslated region. A 3' untranslated region refers to that portion of a gene comprising a DNA segment that contains a polyadenylation signal and any other regulatory signals capable of effecting mRNA processing, mRNA stability, or gene expression. The polyadenylation signal is usually characterized by effecting the addition of polyadenylic acid tracks to the 3' end of the mRNA precursor. Polyadenylation signals are commonly recognized by the presence of homology to the canonical form 5' AATAAA-3' although variations are not uncommon. Examples of suitable 3' regions are the 3' transcribed non-translated regions containing a polyadenylation signal of *Agrobacterium* tumour inducing (Ti) plasmid genes, such as the nopaline synthase (*Nos* gene) and plant genes such as the soybean storage protein genes and the small subunit of the ribulose-1, 5-bisphosphate carboxylase (ssRUBISCO) gene. The 3' untranslated region from the structural gene of the present construct can therefore be used to construct chimeric genes for expression in plants.

The gene constructs of the present invention can also include further enhancers, either translation or transcription enhancers, as may be required. These enhancer regions are well known to persons skilled in the art, and can include the ATG initiation codon and adjacent sequences. The initiation codon must be in phase with the reading frame of the coding sequence to ensure translation of the entire sequence. The translation control signals and initiation codons can be from a variety of origins, both natural and synthetic. Translational initiation regions may be provided from the source of the transcriptional initiation region, or from the structural gene. The sequence can also be derived from the promoter selected to express the gene, and can be specifically modified so as to increase translation of the mRNA.

To aid in identification of transformed plant cells, the constructs of this invention may be further manipulated to include plant selectable markers. Useful selectable markers include enzymes which provide for resistance to an antibiotic such as gentamycin, hygromycin, kanamycin, and the like. Similarly, enzymes providing for production of a compound identifiable by colour change such as *GUS* (β-glucuronidase), or luminescence, such as luciferase are useful.

By "transformation" it is meant the stable transfer of genetic information. The constructs of the present invention can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, micro-injection, electroporation, etc as would be known to those of skill in the art.

Also considered part of this invention are transgenic plants containing the chimeric gene construct of the present invention. Methods of regenerating whole plants from plant cells are known in the art, and the method of obtaining transformed and regenerated plants is not critical to this invention. In general, transformed plant cells are cultured in an appropriate medium, which may contain selective agents such as antibiotics, where selectable markers are used to facilitate identification of transformed plant cells. Once callus forms, shoot formation can be encouraged by employing the appropriate plant hormones in accordance with known methods and the

shoots transferred to rooting medium for regeneration of plants. The plants may then be used to establish repetitive generations, either from seeds or using vegetative propagation techniques.

By "sporophytically expressed pollen coat protein" it is meant a protein synthesised within sporophytic tissue, and via subsequent processing and translocation, is deposited upon the outer surface of the pollen coat. For example, the *Sta* 41-2 and *Sta* 41-9 gene products are expressed within tapetal tissue, and, even though not comprising a signal peptide, these gene products are deposited on the exterior of the pollen coat during development. Furthermore, other gene products comprising signal peptides that are tapetally expressed can be targeted to the pollen coat, e.g. *Sat*ap35 and *Sat*ap44.

By "gametophytically expressed pollen coat proteins" it is meant, proteins that are synthesised within gametophytic tissue, such as microspore, pollen, or ovary cells, and are translocated to the extracellular compartment of these cells. For example, the *PCP1* pollen coat protein is synthesized in the pollen cell and relocated to the pollen coat.

The methods of this invention allow for the localization of a protein or peptide of interest within the extracellular compartment of pollen or stigma cells, including enzymes, receptors, antigens, antibodies, ligands, substrates, inhibitors and peptides which may modify normal male or female reproductive tissues (including but not limited to pollen, microspore, pollen tube, stigma, ovary, or egg) interaction, function, or both. When expressed on the surface of pollen or pistil, the fusion peptides or proteins can be used:

- for the production of either self-incompatible, self-compatible, or self-only-compatible flowers thus providing a novel method for hybrid seed production, plant maintenance, or to prevent outcrossing;
- as sources of immobilized enzymes;

- as novel methods of controlling pests and pathogens; and

These applications will be described in more detail below.

Furthermore, it is contemplated that the linkage of peptides to the pollen coat may also be achieved chemically. This may be achieved by a variety of ways known to those of skill in the art, for example, using lectin concavalin A to covalently link the proteins to sugar residues on the pollen exine, or using tannic acid to covalently link proteins of interest to naturally occurring proteins expressed on the pollen coat. Such methods may either be used to prepare modified coat walls of pollen for the purposes disclosed within this invention, or for evaluating the feasibility, or function of desired proteins of interest located to the extracellular compartment of pollen, prior to designing, preparing and transforming plants with appropriate constructs and vectors leading to the expression of the desired protein.

In accordance with the subject invention, the method for modifying the protein composition of the extracellular compartment of a floral cell of a plant comprises expressing a construct comprising a gene of interest within an anther or stigma cell. The gene of interest encodes a protein, fusion protein or peptide, or a fragment thereof, and this protein or fragment thereof is capable of modifying the composition of the extracellular compartment of the floral cell. By altering the composition of the extracellular compartment of the floral cell, the function, development or use of the floral cell, or the interaction of said floral cell with other cells is modified while possibly maintaining the pollen, and the cells of the stigma, in a viable state. However, it is not necessary that these cells remain viable. For example, expression of a protease on the surface of a stigma cell may or may not kill the stigma cell depending upon the protease selected and the concentration of protease expressed. If the stigma cell is killed by the protease, pollen (especially if expressing protease inhibitor) may still germinate on the surface of the stigma, however, a female sterile plant may be obtained if germination is prevented as a result of protease expression or

disruption of the stigma cells. Similarly, pollen may or may not remain viable following the modification of the extracellular compartment as described herein.

This invention also relates to a method for expressing a protein of interest on the surface of a pollen or pistil cell which includes preparing an expression cassette containing a construct comprising one or more regulatory sequences and a gene of interest which encodes a polypeptide or derivatives thereof, along with motifs that ultimately direct the expression of the protein extracellularly, so that when produced in a transgenic plant the protein is localized extracellularly, that is that the protein is located on the surface of the pollen or pistil cell. Typically, the peptide of interest is a novel protein not normally found on the pollen or pistil cell surface, however, it is also contemplated that it may be desired to modify the composition of the extracellular domain or the abundance or the properties of a native protein within the extracellular compartment using the method of this invention. For example a protein from the following list, which is not to be considered limiting, may be used for the preparation of chimeric constructs:

cysteine protease from *Sitophilus,* (Matsumoto, I., Emori, Y., Abe, K, Arai, S. *J. Biochem.* **121**: 464-476 (1997)).

cysteine protease inhibitor from *Onchocerca* (Lustigman, S., Brotman, B., Huima, T., Prince, A.M. *Mol. Biochem. Parasitol.* **45**: 65-76 (1991)).

oxidases (Plant Molec. Biol. 30:833-837 (1996));

chitinases (Bork C., and Hell. R. Plant Phys. 115: 864 (1997));

invertase (Lorenz, K., Lienhard, S., Sturm. A. Plant Mol. Biol. 28:189-194. (1995));

endo-β-1,4-xylanase (Millward-Sadler S., Davidson K., Hazelwood G., Black G., Gilbert H., Clarke J. *Biochem. J.* **312**: 39-48 (1985));

callase from *Arabidopsis thaliana* (Patent WO 9302197-A);

lipases, for example **triacylglycerol lipase** (EC.3.1.1.3) from *Magnaporthe grisea* (Wu, Bernstein, Darvill, Albersheim Genebank Accession No. AA415091 (1997);

phytase from *Aspergillus fumigatus* (Pasamontes, L., Haiker, M., Wyss, M., Tessier, M., VanLoon, A. *Appl. Environ. Microbiol.* **63**: 1696-1700 (1997);

glucosidases, for example, **glucan 1,3 β-glucosidase** cDNA from *Schizosaccharomyces pombe* (Yoshioka, S., Kato, K., Okayama, H. Genebank Accession No. AB000539 (1997);

endo 1,3-1,4β-glycanase cDNA from *Sinorhizobium meliloti* (York, G., Walker, G. *Mol. Microbiol.* **25**: 117-134 (1997);

N-glycosidase F from *Flavobacterium menigosepticum* (EC. 3.5.1.5.2) (Lemp, D., Haselbeck, Kleb, F., *J. Biol. Chem.* **265**: 1506-15610 (1990);

trypsin inhibitor from *Brassica oleracea* (Williams, D., Kain, W., Broadway, R. *Plant Physiol.* **114**: 747(1997);

caspase eg. ICE cysteine proteases (Thornberry et al. *Nature* **356**: 768-774 (1992);

aspartic protease from *Brassica napus* (D'Hondt, K., Bosch,D., VanDAmme, J., Goethals, M., Vanderkerckhove, J., Krebbers E. *J. Biol. Chem.* **268**: 20884-20891(1993);

lactase (intestinal) (*Biochem. J.* **322**: 491-498 (1997));

cellulases and **xylanases** (Li, X., Chen, H., Ljungdahl, L. *Appl. Environ. Microbiol.* **63**: 628-635 (1997);

fructosyl amino acid oxidases (Yoshida,N., Sakai, Y., Isogai, A., Fukuya, H., Yagi, M., Tani,Y., Kato, N. *Eur.J. Biochem.* **242**:499-505 (1996);

polygalacturonidase (Ruttkowski, E., Ngugen, Q., Gottshalk, M., Jany, K., Loeffler,F., Piepersberg,W., Schuster, E., Gassen, H. (Patent EP 0388593-A);

pectate lyase from *Zea mays* (Turcich, M. P., Hamilton, D.A., Mascarenhas, J.P. *Plant Mol. Biol.* **23**: 1061-1065 (1993);

pectin methylesterase (Turner, L., Kausch, K., Hand. A. Plant Physiol. 111: 652. (1996));

chalcone synthase (Itoh, M., Ichinose, Y., Kato, H., Shiraishi, T., Yamada, T. *Mol. Gen. Genet.* **255**: 28-37(1997);

alginate lyase (Chavagnat, F., Duez, C., Guinand, M.,Potin, P.,Barbeyron, T., Henrissat,B.,Wallach, J.,Ghuysen,J . *Biochem. J.* **319**: 575-583 (1996);

D-amino acid oxidase (Konno, R. *Biochem. Biophys. Acta* in press (1997);

β-glucuronidase (Jefferson, R., Kavanagh, T., Bevan, M. *EMBO J.* **6:** 3901-3907 (1987).

lectins such as avidin (Gope, M., Keinanen, R., Kristo, P., Connely, O., Beattie, W., Zanucki-Schulz, T., O'Malley, B., Kulomaa M. Nucleic Acids Research 15:3595-3606. (1987)),

arabinogalactans (Gerster, J., Allard, S., Robert, L.S. Plant Physiol. 110: 1231-1237. (1996)).

canine parvovirus coat protein (Dalsgaard, K., Uttenthal, A.,Jones, T., Xu, F., Merryweather, A., Hamilton, W., Langeveld, J., Boshuizen, R., Kamstrup, S., Lomonossoff, G., Porta, C.,Vela, C.,Casal, I., Meloen, R., Rodgers, P. *Nature Biotech.* **15**: 248-252 (1997).

nuclease (Hartley, R.W. Biochem 32:5978-5984 (1993)).

calpan (Karcz, S., Podesta, R., Siddiqui, R., Dekaban, G., Strejan, G., Clarke, M. Molec. Biochem. Parasitology 49:333-336 (1991)).

thaumatin (Ruiz-Medrano, R., Jimenez-Moraila, B., Herrera-Estrella, L., Rivera-Bustamante R. Plant Mol. Biol. 20:1199-1202. (1992));

Pin-I and Pin-II, protease inhibitors (Johnson, R., Narvaez, J., An, G., Ryan C., *Proc. Nat. Acad. Sci. USA* **86**: 9871-9875 (1989)).

calmodulin (Heo, W.D. et al. *Proc. Nat. Acad. Sci. USA* **96**: 766-771 (1999)).

aquaporin (Ikeda, S. et al. Science **276**: 1564-1566, (1997)).

phosphatases (Rodriguez, P.L. Plant Mol. Biol. **38**:919-927 (1998)).

proteins involved in wax synthesis (Aarts, M.G et al. Plant Cell **12**:2115-2127 (1995)).

pollen coat proteins (Doughty, J. et al. Plant Cell **10**: 1333-1347 (1998); Stancher, B.S. et al. Plant J. **10**: 303-313 (1996)).

Proteins localized extracellularly can be used to modulate pollen function and for example prevent normal fertilization. The pollen from the genetically modified plant can also be used as a carrier for various polypeptides. This provides a novel protein expression, production and purification system.

In order to obtain a sporophytically expressed pollen coat protein, the tapetal oleosin-like proteins are fused translationally to a polypeptide of interest. For example, which is not to be considered limiting, the nucleotide and deduced amino acid sequences of a tapetal expressed oleosin-like gene Sta 41-9 (Robert , L.S., Gerster, J., Allard, S., Cass, L., Simmonds, J. *Plant J.* **6**:927-933 (1994a)) is used for the preparation of a number of possible translational fusions. The polypeptide of interest can be fused to the C-terminal end of the protein *Sta*41-9 (Figure 3A, see Example 2), or the polypeptide of interest can also be fused internally next to the proteolytic cleavage site observed in the pollen targeted protein. In this latter fusion, the native C-terminal region can be deleted or preserved (Figure 3B and 3C, respectively). It is to be understood that the constructs exemplified in Figures 3A-3C are to be considered examples of a range of possible protein fusions that could be prepared using the *Sta*41-9 gene, and are not intended to limit the scope of this invention in any manner. Furthermore, other expressed proteins disclosed within the prior art (e.g. U.S. 5,652,354; U.S. 5,571,904; U.S. 5,633,438; U.S. 5,545,546; U.S. 5,659,124; WO92/13957; WO97/13401; WO93/25695; CA 2,099,482; CA2,106,718; CA2,165,934) may also be used for translational fusions and are considered within the scope of the present invention.

Also considered within the scope of this invention is the expression of a gene in the tapetum whose product could modify a protein which is subsequently targeted to the extracellular domain of pollen. Similarly, the expression of a gene encoding a protein that is targeted to the extracellular domain of a floral cell, for example, but not limited to, an oleosin-like protein, may be inhibited using methods known within the art, for example but not limited to, antisense. In this manner a reduction of a protein within the extracellular domain of a pollen grain results and modifies pollen stigma interaction.

Gametophytically expressed pollen coat proteins are also used in translational fusions with the polypeptide of interest or this polypeptide is directed to the microspore or pollen coat by transcriptional or translational fusion to a promoter directing pollen

expression. These can be part of the coat of the pollen grain or can be released extracellularly. As an example, which is not to be considered limiting in any manner, a translational fusion is made to the cysteine rich *B. oleoracea PCP1* pollen coat protein (Stanchev, B.S., Doughty, J., Scutt, C.P., Dickinson, H., Croy, R.R.D. *Plant J.* **10**:303-313 (1996)) which is synthesized in the pollen cell and relocated to the pollen coat.

It is also contemplated that a chimeric DNA construct encoding a polypeptide of interest can be prepared so that the polypeptide is synthesised within gametophytic tissue, and released at a latter time, for example within pollen, and released upon pollen germination. In this case, the protein of interest is either fused translationally to *B. napus* pollen polygalacturonase *Sta*44 (Robert, L.S., Allard, S., Gerster, J.L., Cass, L., Simmonds, J. *Plant Mol. Biol.* **23**:1273-1278 (1993)), or fused transcriptionally to the promoter of *Sta*44 G(2) (Hong, H.P., Gerster, J.L., Datla, R.S.S., Albani, D., Scoles, G., Keller, W., Robert, L.S. *Plant Cell Rep.* **16**:373-378 (1997); US patent application 08/577,463). These fusion proteins are then produced within the pollen grain and released upon germination.

The method of this invention can be used for the purposes of altering pollen function; preventing self-pollination, allowing self-pollination; preventing cross-pollination; peptide display, or treatment or control of insect populations. These are discussed in more detail below:

1. **Alter pollen or pistil function.**

Normal pollen function necessitates specific inter- and intra-molecular interactions with constituents of the stigma, style and ovary. Modifying pollen or pistil function, or these interactions could result in the failure or allowance of pollen germination, pollen tube growth and/or fertilization of the egg. Modification can be achieved by specific catalytic enzymes (eg. proteases, glucosidases, glycanases, nucleases, lipases, hydrolases etc.), toxins (eg. *Diphteria* toxin A chain), antibodies, lectins etc. localized

on the surface of the pollen or pistil cell, or released from the pollen, or pollen tube.

As an example of this embodiment, which is not to be considered limiting in any manner, the tapetal expressed Sta 41-9 protein or the gametophytically expressed Sta 44 is fused to a cysteine protease from *Sitophilus,* (Matsumoto, I., Emori, Y., Abe, K, Arai, S. *J. Biochem.* **121**: 464-476 (1997)). It is also contemplated that transcriptional fusions may also be used for protease expression. Once targeted to the microspore/pollen coat, these enzymes digest proteins important to pollen germination or pollen-stigma interactions and therefore prevent pollen germination or pollen tube growth (see Figure 11). It is to be understood that other proteases such as trypsin, chymotrypsin, caspases, sulfhydryl proteases, aspartic proteases or metallo-proteases and the like may also be used. Furthermore, other tapetal or pollen expressed proteins may also be used as the basis for protein fusions as would be evident to one of skill in the art. Similarly, as an example of passively targeting extracellular localization, pollen coat composition could be modified by tapetal proteins, encoded by chimeric gene constructs, which are secreted or released in the anther locule. Other modifying polypeptides such as those mentioned above could also be used in a similar way to affect pollen function (e.g. see Figure 17). For example, which is not to be considered limiting, these chimeric gene constructs may also comprise sequences encoding proteins such as the protease inhibitor from *Onchocerca* (see Figure 15).

As further examples of this embodiment, chimeric gene constructs could comprise the stigma expressed gene encoding the Pis 63 protein fused to the *Sitophilus* protease (see Figure 14) or *Onchocerca* protease inhibitor (see Figure 12). These proteins could be used to modify pollen-stigma interactions or functions. Other proteins, for example glycanase, may also be localized on the surface of the stigma to affect pollen-stigma interactions or functions (see Figure 18).

2 **Permitting hybrid seed production by preventing self-pollination.**

Heterosis (hybrid vigour) corresponds to the increase in productivity and vigour which results from the genetic differences in parental lines. The advantage of growing hybrid crop varieties becomes evident when the benefits from the yield increase due to heterosis significantly outweigh the cost of seed production. Values reported for heterosis in *Brassica napus* seed yield have been greater than 50% (Grant I. and Beversdorf W.D., *Can. J. Genet. Cytol.* **27**:472-478 (1985)). Although heterosis is observed in nearly every crop, the use of hybrids is mostly limited to crops for which there is an economically viable and effective means of pollination control. Many major crops, such as Canola, have small bisexual flowers which render manual emasculation impractical and thus hybrids cannot be produced commercially without using methods of interfering with pollen development and causing male sterility (Williams M.E., *TIBTECH* **13**:344-349 (1995)). Cytoplasmic male sterility (CMS) has been used for this purpose in oilseed rape, but this approach suffers from the breakdown of sterility in certain environments and from undesirable agronomic traits linked to the restorer genes (Feistritzer, W.P. and Kelly, A.F., (eds) *Hybrid Seed Production of Selected Cereal Oil and Vegetable Crops*, FAO (1987)). Genetically engineered nuclear-encoded male sterility may represent a viable alternative approach for pollination control in crops such as Canola (Stefansson, B.R. and Downey, R.K. in: *Harvest of Gold: The History of Field Crop Breeding in Canada*, Slinkard, A.E. and Knott, D.R. (Eds) Saskatoon: University Extension Press (1995)).

Several stages of plant reproduction including gamete development, pollination and fertilization depend on both gametophytic and sporophytic gene expression. Male or female sterility can result from mutations disrupting normal gene expression in haploid (eg. pollen) or diploid cells (eg. tapetum) throughout these developmental stages. Examples of molecular approaches used successfully to generate transgenic male sterile plants have been reported. Such approaches can involve the use of cytotoxic genes (Mariani, C., De Beuckeleer, M., Truettner, J. Leemans, J., Goldberg, R.B. *Nature* **347**:737-741 (1990); Koltunow, A.M., Truettner, J., Cox, K.H., Wallroth, M., Goldberg, R.B. *Plant Cell* **2**:1201-1224 (1990)), antisense versions of essential pollen genes (Muschietti, J., Dircks, L., Vancanney, G.,

McCormick, S. *Plant J* **6**:321-338 (1994); Xu, H., Knox, R.B., Taylor, P.E., Singh, M.B. *Proc. Natl. Acad. Sci. USA* **92**:2106-2110 (1995)) or genes encoding enzymes involved in pollen development (Worrall, D., Hird, D.L., Hodge, R., Paul, W., Draper, J., Scott, R. *Plant Cell* **4**:759-771 (1992)). These approaches have been the subject of patent applications such as WO 90/08828 and WO 92/18625. However, none of these approaches are aimed at modifying the protein composition of the microspore/pollen coat or the interactions of these proteins.

The targeting to the microspore/pollen coat of proteases, antigens, enzymes, inhibitors, or peptide(s) which interact with endogenous or supplemented stigma constituents (eg. stigma expressed protease inhibitors, antibodies, protein binding polypeptides, proteolytic enzymes) modulates the activity of the microspore/pollen coat or stigma (see Figures 13 and 16). This approach modifies self-compatibility or self-incompatibility, or prevents out-crossing (cross-pollination) and results in a "lock and key mechanism" for controlling pollination since only specific pollen germinate on specific stigmas. This lock and key mechanism may function either within the same plant or on separate plants. For example, which is not to be considered limiting, a male plant transformed with the Sta 41-9/cysteine protease from *Sitophilus,* (Matsumoto, I., Emori, Y., Abe, K, Arai, S. *J. Biochem.* **121**: 464-476 (1997)) fusion construct will not self pollinate however, the pollen will germinate on a female plant that has been transformed so that the stigma expresses a specific inhibitor, the *Onchocerca* cysteine protease inhibitor gene (Lustigman, S., Brotman, B., Huima, T., Prince, A.M. *Mol. Biochem. Parasitol.* **45**: 65-76 (1991)). The resulting seed of such a cross is hybrid and this represents a novel molecular approach to hybrid seed production. However, as would be evident to one of skill in the art, other protein/protein or protein/substrate interactions such as, but not limited to, protease/inhibitor, or activator/repressor, or receptor/ligand, or nuclease/inhibitor combinations may be used in this application. Maintaining either parental line is possible through the use of exogenously applied moderators, such as protease inhibitor solutions as required.

This approach can also be used to prevent out-crossing (cross-pollination). In this example, the same plant has both the pollen coat protease and a stigmatic inhibitor of the protease thereby allowing self fertilization (Figure 13). Pollen grains with the protease will be unable to germinate on any other plant. Furthermore, the reverse approach is feasible, plants could be generated that express the protease in the stigma thus allowing only pollen with the specific protease inhibitor to germinate (Figure 16). The utility of these approaches is the control of pollen flow among transgenic cultivars in plant species prone to outcrossing for eg. Canola. This may help diminish environmental concerns with respect to the release of transgenic plants, and may serve to reduce the risk of outcrossing between different transgenic lines, or cultivars.

It is also contemplated that localization of a tapetal oleosin-like/anti-*S*-locus glycoprotein antibody fusion to the pollen coat could be used to disrupt normal pollen or pistil development For example, an antibody to a *S*-locus glycoprotein could be raised and the immunoglobulin heavy chain and light chain variable regions fused into a single chain antibody fragment (ScFv). This fragment could be cloned and expressed on the pollen coat as described in Examples 1-3. When expressed on the pollen coat of a self-incompatible *Brassica* species for example, this antibody could interfere with the normal interactions with the stigma and abolish self-incompatibility. Other examples of polypeptides which could also disrupt or improve normal pollen or pistil development include: lectins such as avidin and arabinogalactan proteins (Gerster, J., Allard, S., Robert, L.S. Plant Physiol. 110: 1231-1237 (1996)).

3. **Targeting proteins or peptides to the pollen coat for the purposes of peptide display, for protein production and utilization.**

The display of a protein on the surface of a pollen grain would permit a ready source of the protein for further purification or for utilization, for example as an immobilized enzyme. It is conceived that the construct employed for protein production further comprises a site that permits cleavage of the protein from the rest of the fusion protein.

Such a site may be a proteolytic site and susceptible to cleavage using a protease, or a site susceptible to chemical cleavage. Proteins or peptides produced in plants have the advantage of being properly glycosylated as opposed to prokaryotic expression systems. Any peptide which could be produced by this method may be selected for use in this application, including therapeutic, or nutritive peptides such as tritrpticin and leptin or any other useful polypeptide such as avidin, interleukin, interferon etc..

Immobilized enzymes may also be prepared using the method of this invention. For example, the enzyme β-glucuronidase (GUS) when targeted to the pollen coat could be utilized as an immobilized enzyme. Other examples of enzymes which could be attached to the pollen coat include invertase, xylanase, β-1,3-glucanase, cysteine protease *(Sitophilus),* cysteine protease inhibitor (*Onchocerca),* oxidases, chitinases, invertase, endo-β-1,4-xylanase, callase, triacylglycerol lipase, phytase, glucan 1,3 β-glucosidase, endo 1,3-1,4β-glycanase, N-glycosidase, trypsin inhibitor, caspase (e.g. ICE cysteine proteases), aspartic protease lactase (intestinal), cellulases, xylanases, fructosyl amino acid oxidases, polygalacturonidase, pectate lyase, pectin methylesterase, chalcone synthase, alginate lyase, D-amino acid oxidase, β-glucuronidase.

Tapetal oleosin-like protein fusions may be used to produce, utilize or purify recombinant polypeptides. For example, the peptide tripticin can be produced on the surface of pollen and used directly as a bacteriostatic agent. Alternatively, the recombinant peptide can be proteolytically cleaved by introducing a cleavage site, such as the one used by thrombin, between the tapetal oleosin-like protein and the peptide of interest. Other peptides which could be produced in this way include, for example, leptin fragment, lectin (e.g. avidin), arabinogalactans, canine parvovirus coat protein, thaumatin, Pin-I and Pin-II, protease inhibitors.

4. **Antigen delivery for antibody production**.

To generate anti-peptide or anti-protein antibodies, the peptides are often prepared by chemical synthesis using solid phase techniques (Merrifield, R. *Science* **85**: 2149-2154 (1963)) and coupled to a carrier. Since many small peptides (haptens) are not highly immunogenic, they require a means of increasing their antigenicity such as chemical coupling to keyhole limpet haemocyanin (KLH), or bovine serum albumin (BSA). By expressing a peptide on the surface of, or within pollen grains, these peptides can be released or presented directly to the animal immune system. The benefits include multiple copies of the antigen displayed on the surface of each pollen grain, the elimination of chemical coupling to carrier molecules, the production of large quantities of pollen and therefore antigen, and the possibility to administer the immunogen orally or nasally and therefore stimulate the mucosal immune system. The large size of the pollen grain may also alleviate the need for adjuvants and therefore be useful in immunization.

This approach is also an economical means of producing and presenting oral vaccines and therapeutic agents, since plants are not known to be contaminated with any animal viruses or pathogens. Recombinant proteins and therapeutics may be expressed in transgenic plants and packaged on intact pollen grains with little processing or purification in some cases. Irradiation of pollen grains prior to administration or use could eliminate the possibility of pollen escape.

Antigens for antibody or vaccine production may also be prepared using the method of this invention. For example, the antigen or vaccine could be fused to the tapetal oleosin-like protein and pollen coated with this fusion protein could be administered for example: intradermally, intramuscularly, intraperitoneally, intravenously, subcutaneously and nasally (nasal spray). Any protein or peptide now used for the production of vaccines could be utilized in this way, for example canine parvovirus (Dalsgaard, K., Uttenthal, A., Jones, T., Xu, F., Merryweather, A., Hamilton, W., Langeveld, J., Boshuizen, R., Kamstrup, S., Lomonossoff, G., Porta, C.,Vela, C.,Casal, I., Meloen, R., Rodgers, P. *Nature Biotech.* **15**: 248-252 (1997).

This approach can also be used to raise antibodies to poorly immunogenic antigens such as small peptides due to the method of presentation on the pollen grain.

5. **Treatment or control of insect populations.**

Beneficial insects such as honeybees can be beneficially treated by providing transgenic pollen comprising antibiotics or food supplements (eg. synthetic proteins or peptides rich in insect-essential amino acids, especially the aromatic amino acids). The treatment of harmful, destructive or phytophagous insects such as pollen beetles with antifeedants or antibiotics (protease inhibitors, Bt toxins, lectins) represents a novel and efficient control method of these insects. Examples of protease inhibitors which may be used in this manner include the serine class of protease inhibitors, Pin-I and Pin-II, (Johnson, R., Narvaez, J., An, G., Ryan C., *Proc. Nat. Acad. Sci. USA* **86**: 9871-9875 (1989)), cysteine protease inhibitor onchocystatin (OV7; Lustigman S et al. Mol. Biochem. Parasitol. 45:65-76 (1991)). The effects of the inhibitors can be measured by monitoring weight loss of insects feeding on modified pollen. An alternative strategy is to employ pollen coated with protease inhibitor inducing factors (PIIF) which induce the systemic induction of protease inhibitors and defensive compounds in distal organs of plants (McGurl, B.F., Pearce, G., Orozco-Cardenas, M., Ryan, C.A. *Science* **255**:1570-1573 (1992)). In a similar manner, pollen coat targeting of insecticidal toxins from *Bacillus thuringiensis* (Bt toxins) in plants such as corn (*Zea mays*) which sheds high quantities of pollen over foliar, silk, and floral surfaces represents a novel method to deliver insecticidal proteins. This method of delivery is also useful for the dissemination of antifungal, antiviral, and antibacterial peptides and proteins over the vegetative and floral surfaces of plants. Such proteins could consist of pectinases, oxidases, chitinases.

The present invention will be further illustrated in the following examples. These examples are for illustrative purposes only, and should not be used to limit the scope of the present invention in any manner.

Examples

The following sequences are used in order to exemplify the present invention:

SEQ ID NO: 1 is oligonucleotide sequence (KSB-3) corresponding to the plus strand of the KSB adapter.

SEQ ID NO: 2 is oligonucleotide sequence (KSB-4) corresponding to the minus strand of the KSB adapter.

SEQ ID NO: 3 is the nucleotide sequence of the translational fusion in plasmid TOG-1. This fusion consists of the 5' upstream and coding sequence from the *Brassica napus* genomic clone *Sta* 41 G(10) which encodes the *Sta* 41-9 tapetal oleosin-like protein fused to the *E. coli* β-glucuronidase coding region from plasmid pBI 101.1 (Clontech). The upstream region was shown to regulate tapetal-specific expression in transgenic *Brassica napus* plants (Hong, H.P., Ross, J.H.E., Gerster, J.L., Rigas, S., Datla, R.S.S., Hatzopoulos, P., Scoles, G., Keller, W., Murphy, D.J., Robert, L.S. *Plant Mol. Biol.* **34**:549-555 (1997); US patent application 08/595,937).

SEQ ID NO: 4 is the nucleotide sequence of the translational fusion in plasmid TOP-1. This fusion consists of the 5' upstream and coding sequence from the *Brassica napus* genomic clone *Sta* 41 G(10) which encodes the *Sta* 41-9 tapetal oleosin-like protein fused to the *Sitophilus zeamais* SCP protease coding region (Matsumoto, I., Emori, Y., Abe, K., Arai, S. *J. Biochem.* **121**:464-476 (1997)).

SEQ ID NO: 5 is the nucleotide sequence of the translational fusion in plasmid TOPI-1. This fusion consists of the 5' upstream and coding sequence from the *Brassica napus* genomic clone *Sta* 41 G(10) which encodes the *Sta* 41-9 tapetal oleosin-like protein fused to the *Onchocerca volvulus* protease inhibitor coding region (Lustigman, S., Brotman, B., Huima, T., Prince, A. M. *Mol. Biochem. Parasitol.* **45**:65-76 (1991)).

SEQ ID NO: 6 is oligonucleotide sequence (BKX-1) corresponding to the plus strand of the BKX 12 adapter.

SEQ ID NO: 7 is oligonucleotide sequence (BKX-2) corresponding to the minus strand of the BKX 12 adapter.

SEQ ID NO: 8 is the nucleotide sequence of the translational fusion in plasmid SPF-1. This fusion consists of the 5' upstream and coding sequence from the *Brassica napus* genomic clone GPIS363 which encodes a gene highly expressed in the stigma fused to the *Sitophilus zeamais* protease coding region.

SEQ ID NO: 9 is the nucleotide sequence of the translational fusion in plasmid SPIF-1. This fusion consists of the 5' upstream and coding sequence from the *Brassica napus* genomic clone GPIS363 which encodes a gene highly expressed in the stigma fused to the *Onchocerca volvulus* protease inhibitor coding region.

SEQ ID NO: 10 is oligonucleotide sequence SLG26 (7).

SEQ ID NO:11 is oligonucleotide sequence SLG 26 (8).

SEQ ID NO:12 is the nucleotide sequence of the translational fusion in plasmid SPOV-1. This fusion consists of the CaMV double enhancer promoter fused to the partial coding region of *Brassica napus* cDNA clone SLG_{WS1} including the signal peptide fused to the *Onchocerca volvulus* protease inhibitor coding region.

SEQ ID NO:13 is oligonucleotide sequence (EXK-1) corresponding to the plus strand of the EXK 12 adapter.

SEQ ID NO:14 is oligonucleotide sequence (EXK-2) corresponding to the minus strand of the EXK 12 adapter.

SEQ ID NO:15 is the nucleotide sequence of the translational fusion in plasmid POV-1. This fusion consists of the 5' upstream and the partial coding region including the signal peptide of *Brassica napus* genomic clone Sta 44 G(2) which encodes a pollen expressed polygalacturonase gene (Robert, L.S., Allard, S., Gerster, J.L., Cass, L., Simmonds, J. *Plant Mol. Biol.* **23**:1273-1278 (1993); Hong, H.P., Gerster, J.L., Datla, R.S.S., Albani, D., Scoles, G., Keller, W., Robert, L.S. *Plant Cell Rep.* **16**:373-378 (1997); US patent application 08/577,463) fused to the *Onchocerca volvulus* protease inhibitor coding region.

Example 1: Isolation of *Brassica napus* tapetal oleosin-like gene.

The cDNA clones *Sta* 41-2 and *Sta* 41-9 encoding tapetal oleosin-like proteins were isolated by differential screening of a flower cDNA library from *Brassica napus* (Robert, L.S., Gerster, J.L., Allard, S., Cass, L., Simmonds, J., *Plant J.* **6**:927-933 (1994)). The genomic clone *Sta* 41G(10) corresponding to cDNA clone *Sta* 41-9 was also isolated and the region upstream of the coding region shown to direct expression of a marker gene to the tapetum of transgenic *Brassica napus* plants (Hong, H.P., Ross, J.H.E., Gerster, J.L., Rigas, S., Datla, R.S.S., Hatzopoulos, P., Scoles, G., Keller, W., Murphy, D., Robert, L.S. *Plant Mol. Biol.* **34**:549-555 (1997); US patent application 08/595,937).

The genomic clone *Sta* 41G(10) is used for the construction of translational fusions to polypeptides of interest for targeting to the pollen coat. Other tapetal oleosin-like genes are also known (Ross, J.H.E., Murphy, D.J. *Plant J.* **9**:625-637 (1996); Ruiter, R.K., Van Eldik, G.J., Van Herpen, R.M.A., Schrauwen, J.A.M., Wullems, G.J. *Plant Cell* **9**:1621-1631 (1997)) and can be used for gene fusions aimed at targeting polypeptides to the pollen coat.

Example 2: Fusion of a *Brassica napus* tapetal oleosin-like gene to the *E. coli* β-glucuronidase gene.

The *Sta* 41 G(10) *Sac* I subclone mp 101 is digested with *Bam* HI and *Hind* III releasing a fragment containing the 5' upstream region of the tapetal oleosin-like gene and cloned into the *Bam* HI and *Hind* III sites of pBSK+ (Stratagene) to generate plasmid BH-1. Plasmid BH-1 is digested with *Nde* I, blunt ended with the Klenow fragment of DNA polymerase I and religated, effectively destroying the *Nde* I site and generating plasmid NB-6. Plasmid NB-6 is digested with *Bgl* II and *Kpn* 1 and used to replace the *Bgl* II and *Kpn* 1 fragment of mp 101. This effectively reconstructs the *Sta* 41 G(10) *Sac* I subclone (without the *Nde* I site within the promoter while preserving the *Nde* I site just upstream of the stop codon) and generates plasmid KB-1 (Figure 4(A)). The *Sta* 41G(10) *Sac* I subclone is then cloned into pGEM7Z (Promega) to give plasmid SS-4. Plasmid SS-4 is digested with *Nde* I and ligated to the adaptor KSB (SEQ ID NOs: 1 and 2) generating plasmid OFC-1 (Figure 4(B)). This double stranded adaptor possesses *Nde* I overhangs and encodes the restriction sites *Kpn* I, *Sac* I and *Bam* HI. The *Bam* HI fragment of OFC-1 containing the tapetal oleosin-like gene (promoter and coding sequence) is translationally fused to the GUS coding region (followed by the Nos terminator) of pBI 101.1 (Clontech) to produce plasmid TOG-1 (Figure 5; SEQ ID NO:3).

Example 3: Fusion of the as *Brassica napus* tapetal oleosin-like gene to the *Sitophilus* protease gene.

A fragment of a cysteine protease from *Sitophilus,* from plasmid pSCPc1, is amplified by PCR (polymerase chain reaction). This fragment corresponds to the SCPc1 cDNA fragment cloned into pBSK- (Matsumoto, I., Emori, Y., Abe, K, Arai, S. *J. Biochem.* **121**: 464-476 (1997)). The oligonucleotide primers used in the PCR reaction are:

forward primer: P1: 5' GCGC**GGATCC**TTGCCTGATACTGTTGAC

and reverse primer:

P2: 5' GCGC**GAATTCAAGCTT**CTAAACCAAAGGATAACTAGC.

These primers permit the amplification of the mature cysteine protease coding sequence and introduce a *Bam* HI site (bold) at the 5' end of the amplified DNA fragment and *Eco* RI and *Hind* III sites (bold) at the 3' end.

The PCR fragment is digested with *Bam* HI and *Hind* III and subcloned into the *Bam* HI and *Hind* III sites of pGEM 4Z (Promega) generating plasmid pSCPc1BH. Plasmid pSCPc1BH is digested with *Bam* HI and *Hind* III and ligated into the *Bam* HI and *Hind* III sites of pGEM 7Z generating plasmid SCP-2. Plasmid SCP-2 is digested with *Bam* HI and *Sma* I and the fragment containing the protease coding sequence ligated into the *Bam* HI and *Sma* I of Binter (this corresponds to the binary vector Bin 19 (Bevan, M. *Nucl. Acids Res.* **12**:8711-8721 (1984)) into which the nopaline synthase terminator polyadenylation signal is subcloned as a *Sac* I and *Eco* RI fragment) generating plasmid BS-2. The *Bam* HI fragment of OFC-1 containing the tapetal oleosin promoter and coding sequence referred to in Example 2 is ligated into the *Bam* HI site of BS-2 generating a translational fusion between the tapetal oleosin-like gene and the *Sitophilus* protease gene in plasmid TOP-1 (Figure 6; SEQ ID NO:4). The *Eco* RI/*Bam* HI fragment containing the *Sitophilus* protease/nopaline synthase polyadenylation signal of plasmid BS-2 was also cloned into plasmid pHS723 (Datla, R.S.S. Plant Biotechnology Institute, Saskatoon Canada) to generate binary vectors with the GUS/NPTII fusion.

Example 4: Fusion of a *Brassica napus* tapetal oleosin-like gene to the *Onchocerca* cysteine protease inhibitor gene.

Plasmid pGEXOV7 (Lustigman, S., Brotman, B., Huima, T., Prince, A.M. *Mol. Biochem. Parasitol.* **45**: 65-76 (1991)) containing the cDNA clone coding for the *Onchocerca* PI is digested with *Eco* RI releasing a 582 bp fragment which is blunt ended by filling in with the Klenow DNA polymerase I fragment and subcloned into the *Sma* I site of pGEM 4Z generating plasmid pGEMOV7. This fragment contains the coding sequence for the mature *Onchocerca* PI. Plasmid pGEMOV7 is digested with *Bam* HI and *Eco* RI and ligated into the *Bam* HI and *Eco* RI sites of pGEM 7Z

to generate plasmid OV-71. Plasmid OV-71 is digested with *Sac* I and ligated into the *Sac* I of Binter generating plasmid BO-3. The *Bam* HI fragment of OFC-1 containing the tapetal oleosin promoter and coding sequence referred to in Example 2 is ligated into the *Bam* HI site of BO-3 generating a translational fusion between the tapetal oleosin-like gene and the *Onchocerca* PI gene of plasmid TOPI-1 (Figure 7; SEQ ID NO:5). The *Eco* RI/*Hind* III fragment of plasmid BO-3 containing the *Onchocerca* protease inhibitor/nopaline synthase polyadenylation signal was also cloned into pHS723 to generate binary vectors with the GUS/NPTII fusion.

Example 5: Fusion of the *Brassica napus* GPIS363 gene to the *Sitophilus* protease.

A fragment of genomic clone GPIS363 containing the promoter and the gene coding region was cloned into pGEM 4Z resulting in plasmid Bg2. Plasmid Bg2 was digested with *Kpn* I and *Xba* I, and the insert cloned into the corresponding sites of pGEM 7Z to yield plasmid KX-1 (Figure 8). Plasmid KX-1 was digested with *Xho* I and ligated to the BKX 12 adaptor (SEQ. ID NOs: 6 and 7) introducing additional *Bam* HI, *Kpn* I and *Xba* I sites within the 3' coding region and resulting in plasmid XX-1. The *Bam* HI fragment of plasmid XX-1 containing the GPIS363 promoter and partial coding region was cloned into BS-2 (Figure 6) resulting in a translational fusion to the SCP protease in plasmid SPF-1 (Figure 8; SEQ ID NO: 8).

Example 6: Fusion of the *Brassica napus* GPIS363 gene to the *Onchocerca* protease inhibitor.

The *Kpn* I fragment of plasmid XX-1 described above containing the GPIS363 promoter and partial coding region was cloned into BO-3 (Figure 7) resulting in a translational fusion to the OV7 protease inhibitor in plasmid SPIF-1 (Figure 8; SEQ ID NO: 9).

Example 7: Fusion of the Cauliflower Mosaic Virus (CaMV) 35S promoter to the *Brassica napus* SLG_{WS1} signal peptide and the *Onchocerca* protease inhibitor.

The region coding for the signal peptide of the *Brassica napus* SLG_{WS1} gene was obtained by PCR with plasmid SLG 26 using oligonucleotide primers SLG 26(7) and SLG 26(8) (SEQ ID NOs: 10 and 11). These primers introduced respectively *Sac* I and *Kpn* I sites flanking the signal peptide. The signal peptide was digested with *Sac* I and *Kpn* I, and introduced into the *Sac* I and *Kpn* I sites of pGEMOV7 to yield plasmid SP-1 (Figure 9). The translational fusion between the SLG_{WS1} signal peptide and the *Onchocerca* protease inhibitor OV7 was then cloned into the *Sac* I and *Bam* HI sites between the CaMV 35S promoter and polyadenylation signal of vector pFF19 (Timmermans, M.C.P., Maliga, P., Vieira, J., Messing, J. *J. Biotechnol.* **14**:333-344 (1990) to yield plasmid SPFF-1. The construct containing the CaMV 35S double enhancer promoter/SLG_{WS1} signal peptide/*Onchocerca* protease inhibitor OV7/CaMV 35S polyadenylation signal was introduced into the plant binary transformation vector pHS723 (Datla, R.S.S. Plant Biotechnology Institute, Saskatoon Canada) as an *Eco* RI/*Hin*d III fragment to generate the vector SPOV-1 (Figure 9; SEQ ID NO:12). A similar vector called COV-1 containing the signal peptide from the OV7 protease inhibitor instead of the SLG_{WS1} signal peptide was also constructed and used for plant transformation.

Example 8: Fusion of the *Brassica napus* pollen polygalacturonase Sta 44G(2) promoter and signal peptide to the *Onchocerca* protease inhibitor.

Plasmid HP-9 containing the Sta 44G(2) promoter and signal peptide was digested with *Sph* I and ligated to EXK-12 (SEQ ID NOs: 13 and 14) introducing additional *Eco* RI, *Xba* I and *Kpn* I sites downstream of the signal peptide and yielding plasmid SS-1 (Figure 10). Plasmid SS-1 was digested with *Kpn* I and the fragment containing the Sta 44G(2) promoter and signal peptide was cloned into plasmid BO-3 (Figure 7) resulting in a translational fusion to the OV7 protease inhibitor and producing plasmid POV-1 (Figure 10; SEQ ID NO: 15).

Example 9: Covalent linkage of enzymes and proteins to the pollen coat.

Pollen grains may be conjugated to a protein of interest, for example, the enzyme horseradish peroxidase, the protease papain, or the protease inhibitor potato multicystatin, through conjugation to sugar residues on the exine. However, residues of the protein may also be modified prior to conjugation.

Pollen grains from opened flowers of *Brassica napus* cv. Westar are collected and "activated" by suspension in phosphate-buffered saline (PBS) solution containing 10 mM Ca^{+2}, 10 mM Mn^{+2}, 10 mM Mg^{+2} , 0.5% Triton X-100, and 10 μg/mL Concanavalin A (Sigma C2010) for one hour at room temperature. Pollen grains are washed repeatedly in PBS solution by centrifugation at 800 x g in 1.5 ml centrifugation tubes for 5 minutes. Conjugation of purified enzymes (e.g. horseradish peroxidase, papain, or potato multicystatin, a cysteine protease inhibitor) is performed by incubating the selected protein at 50 μg/mL in PBS solution with the activated pollen grains for 1 hour at room temperature. Pollen grains are then washed five times with PBS solution and centrifuged for five minutes.

Germination of pollen grains is evaluated by plating isolated pollen grains on 1 mL of filter sterile Brewbaker medium (10% (w/v) sucrose, 100ppm H_3BO_3, 300 ppm $Ca(NO_3)_2.4H_2O$, 200 ppm $MgSO_4.7H_2O$, 100 ppm KNO_3) in sterile petri dishes 15 x 60 mm diameter at 25 °C for 24 hours. The percentage of germinating pollen grains is determined under an inverted microscope, in the presence or absence of proteases or protease inhibitors.

Detection of the conjugated enzymes is measured by placing the pollen grains in enzyme substrate solutions. For horseradish peroxidase, pollen grains are incubated in 50 mM sodium acetate pH 5.0 containing 0.01% H_2O_2, 200 ug/mL amino ethyl carbazole, and the blue pollen grains were recorded under an inverted microscope. Papain activity is recorded on conjugated pollen grains by incubating pollen grains in microtitre plates containing the chromogenic substrate Pyr-Phe-Leu-pNA at 2 mM in Tris.Cl pH 6.5 at 37°C for 30 min and reading the absorbance of the substrate solution at 410 nm in a microtitre plate reader. Papain inhibitory activity is measured on

conjugated pollen grains by incubating pollen grains in a solution containing 10 μg/mL papain in Tris.Cl at pH 6.5 for 10 minutes at 37°C, and then adding the chromogenic papain substrate Pyr-Phe-Leu-pNA at 2 mM for 30 minutes at 37°C. The reactions are quantitated by measuring the absorbance at 410 nm.

Immune response of conjugated proteins to pollen grains is quantified by washing fresh pollen grains of canola with PBS solution for 5 minutes and centrifugation at 800 xg . Pollen grains are suspended in PBS at 5% (vol/vol) and an equal volume of freshly prepared 0.005% tannic acid solution is added and mixed. This mixture is incubated at 37°C for 15 minutes with gentle agitation, before removing the tannic acid solution by centrifugation. The pollen grains are incubated in either purified papain, horseradish peroxidase, or potato multicystatin in PBS solution for 15 minutes at 37 C with gentle shaking. The pollen grains are then washed in PBS by centrifugation three times prior to immunization of mice. Pollen grains coated with either antigen are used to immunize Balb/c mice. Approximately 100μg of coated pollen grains were suspended in Freund's incomplete adjuvant (100μL) and used to inject into the foot pads of mice. A boost is administered after 10 days with the same concentration of antigen, and the same route of injection. Trial bleeds are examined for the titre of antibodies specific for the antigen coated on the surface of pollen grains, and compared to pre-immune serum.

Example 10: Cysteine protease inhibitor retains specificity when expressed as a fusion protein.

To demonstrate that the protease inhibitor from *Onchocera vovulus* (Lustigman, S., Brotman, B., Huima, T., Prince, A. *Mol. Biochem. Parasitol.* 45: 65-76 (1991) is capable of normal function as a fusion protein, it is cloned into the phagemid pCantab 5E (Pharmacia Inc.) and expressed on the surface of filamentous phage as a fusion to the gene 3 phage coat protein.

A *Sac*I and *Nco* I digest of the coding region of the protease inhibitor clone OV7 (Lustigman et al 1991), is ligated to the pCantab 5E vector (Pharmacia Biotech) Sac I and Nco I sites. This phagemid is transformed into *E. coli* TG1 cells by electroporation. An overnight culture of 10 μL of these cells are grown in 5mLs of SOBAG medium containing 10 μl of Carbenicillin (50 mg/ml) at 28°C. The next day, 0.5 mLs of this culture is used to inoculate 25 mLs of 2xYT medium, containing 50 μL of Carbenicillin (50mg/mL) and 1.56 mLs of 2M glucose and grown for 1.25 hrs at 37°C, with shaking at 250 rpm. At this point 62.5 μL of helper phage strain M13K07 (9.0 x 10 pfu/mL) is added and the cells were incubated at 37°C for 30 min with shaking at 150 rpm, and for another 30 min at 250 rpm. Recombinant phage particles are harvested by centrifugation and PEG precipitation. Phage are used in an ELISA assay to determine the specificity of binding to the cysteine protease papain, the serine protease trypsin, and to rabbit polyclonal antibodies directed against a potato cysteine protease inhibitor which also recognize the native OV7 protease inhibitor (anti-OV7 antibodies). Wells of a Nunc microtitre plate (maxisorb) are coated with either papain, anti-cysteine protease inhibitor antibodies, trypsin, or anti M13 antibodies in 100 μL Na_2CO_3 buffer.

After incubating the phage indicated (OV7-those displaying the cysteine protease inhibitor on their surface, M13-wild type or control phage not displaying any foreign protein, and a no phage control) for 2 hrs, the wells were washed in PBS with 0.5% Tween-20. Phage particles which bound to the antigens were detected with a 1:5000 dilution of anti-M13 antibody/HRP conjugate (Pharmacia). Detection was with the HRP substrate ABTS solution (Pharmacia) for 30 minutes and absorbance was measured at 410 nm.

The results (Table 1) demonstrate that the phage displaying the OV7 protease inhibitor clearly bind specifically to the cysteine protease papain and to the anti-cysteine protease inhibitor antibodies at high levels. The OD values for the negative control phage binding (either M13 phage not expressing the protease inhibitor bound

to papain, or the phage expressing the cysteine protease inhibitor bound to trypsin a serine protease) are very low.

Table 1

Antigen coated in well	Phage Added	OD_{410} *	Result
papain	OV7	0.788	specific binding of OV7-papain
papain	M13	0.043	no binding of M13-papain
papain	none	0.016	no background
none	OV7	0.125	very little OV7-plate interaction
anti-OV7 antibodies	OV7	0.451	specific binding of OV7-antibodies
anti-OV7 antibodies	M13	0.026	no interaction M13-antibodies
anti-OV7 antibodies	none	0.024	no background
trypsin	OV7	0.037	no OV7-trypsin interaction
trypsin	M13	0.026	no M13-trypsin interaction
anti-M13 antibodies	M13	0.326	positive control

*Value for Phage Diluted 1 in 100

Phage display demonstrates that the cysteine protease inhibitor OV7 is capable of functioning as a fusion protein when bound to the phage coat protein 3 and phage particles. This protease inhibitor is capable of binding specifically to papain (cysteine protease) and does not bind to trypsin (serine protease).

It has also been demonstrated that proteases can be expressed on the surface of bacteriophage and that they too can function as fusion proteins (Corey,D.R., Shiau, A.K, Yang, Q., Janawski, B.A., Craik, C.S. (1993) Trypsin display on the surface of bacteriophage. Gene 128: 129-134).

Example 11 : Effect of a cysteine protease papain, a cysteine protease inhibitor onchocystatin, and a protein synthesis inhibitor cycloheximide on the germination of tobacco pollen *in vitro*.

Pollen from *Nicotiana tabacum* cv. Delgold was harvested by placing 5 dehiscent anthers in 1 ml of sterile Brewbaker and Kwack medium (Brewbaker, J., Kwack, B. *Amer. J. Bot.* **50**: 859-865 (1963)) containing various concentrations of cylcoheximide, or papain, or casein. The medium consisted of 10% w/v sucrose, 100 ppm H_3BO_3, 300 ppm Ca $(NO_3)_2 4H_2O$, 200 ppm $MgSO_4 7H_2O$, and 100 ppm KNO_3 with either 42 μM papain (twice crystallized, Sigma), with or without 30 μM recombinant onchocystatin, or 71 μM cycloheximide (Sigma), or 1mg/ml casein. Anthers were vortexed in medium for 30 sec followed by centrifugation at 2,000 rpm for 2 min. Pollen was cultured in 50 μl drops in sterile 15 x 60 mm petri dishes at 25 °C. The percentage of pollen grains germinated after 3 h of culture was measured and the average pollen tube length was recorded using an ocular micrometer on an inverted microscope. The protease papain at 42 μM inhibited the germination and stunted the growth of pollen tubes. The addition of 30 μM of the protease inhibitor onchocystatin (OV7) to 42 μM papain restored the germination and pollen tube growth. Cycloheximide at 71 μM also inhibited the germination and elongation of pollen tubes *in vitro* (Table 2, Figure 19).

Table 2

Treatment	**Pollen Germination**	**Average Pollen Tube Length**
Casein (control)	74%	95 μm
Papain	56%	28 μm
Papain+Onchocystatin	69%	80 μm
Cycloheximide	27%	15 μm

These *in vitro* germination assays demonstrate that pollen germination can be affected by enzymes and antibiotics. Furthermore, the reduction in pollen germination caused by a protease can be restored by a protease inhibitor.

Example 12: *Nicotiana tabacum* transformation.

Plant transformation vectors were introduced separately into *Agrobacterium tumefaciens* strain EHA 105 following the protocol supplied with the Pharmacia *Agrobacterium* cells (product: #27-1535). To prepare the *Agrobacterium* competent cells, 5 ml of YEP medium (10 g yeast extract, 10 g peptone, 5 g sodium chloride per liter, pH 7.0) with 150 µg/ml rifampicin and 100 µg/ml gentamycin was inoculated with a loopful of a glycerol stock of *Agrobacterium tumefaciens* and cultured at 28°C by shaking at 250 rpm approximately 15 h. Two ml of the culture was added to 50 ml of fresh YEP medium and grown at 28°C to an O.D. of 0.5-1.0 at 600 nm. The culture was then chilled on ice for 10 min and centrifuged at 5,000 rpm. The cells were resuspended in 1 ml of cold 20 mM $CaCl_2$. These competent cells were dispensed into pre-chilled 1.5 ml Eppendorf tubes in 100 μL aliquots and frozen at -80°C.

The *Agrobacterium* EHA 105 cells were transformed as follows. One μg of uncut plasmid DNA in water was added to 100 μL of *Agrobacterium* competent cells and incubated on ice for 30 min. The cells were then frozen in liquid nitrogen and thawed quickly at 37°C for 5 min and 1 ml of YEP medium was added to the cell/DNA mixture and incubated at 28°C for 2 h with gentle shaking (100 rpm). Cells were then centrifuged in a microfuge for 30 s, the supernatant was poured out and the pellet resuspended in the remaining supernatant (50-100 μl). The resuspended cells were spread on a YEP plate with 25 μg/ml chloramphenicol and 50 μg/ml kanamycin, and incubated at 28°C for 2-3 days.

Plasmid DNA from individual *Agrobacterium* colonies was digested and analyzed by agarose gel electrophoresis to verify the integrity of the vector. Individual colonies which contained the desired recombinant plasmid were selected and grown overnight in 10 ml LB medium (10 g/L bacto-tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.0) with 25 μg/ml chloramphenicol and 50 μg/ml kanamycin. One ml of overnight culture was centrifuged at 13,000 rpm for 5 min and the cells resuspended in MMO medium (4.6 g/L MMO, GIBCO BRL) to an O.D. of 0.1.

Agrobacterium-mediated transformation of tobacco cv. Delgold was performed as follows. Pieces of fresh young tobacco leaves were sterilized 1-2 min in 70% ethanol, 5 min in Javex and then rinsed in sterile water four 2 min 3 times. Leaf discs were obtained with a 5 mm cork borer. Leaf discs were transferred to a dish containing the *Agrobacterium* cell suspension and placed at 25 °C, 16 h light/8 h dark with lights to 70-100 μE for 2-3 days. The co-cultivated discs were transferred to TTK plates (4.56 g/L MMO, 1.0 mg/L benzyl adenine (BA), 0.1 mg/L α-naphthaleneacetic acid (NAA), 3% sucrose, pH 5.8; 300 μg/ml timentin and 25 μg/ml kanamycin added after autoclaving) and incubated at 25 °C, 16 h light/8 h dark with lights to 70-100 μE for 2 weeks. Regenerated shoots were transferred to Magenta GA-7 vessels containing B5 rooting medium (23.2 g/L Gamborg's B5 medium (GIBCO BRL), 7.5 g/L phytagar, pH 5.7; 300 μg/ml timentin and 100 μg/ml kanamycin added after autoclaving). Once a good root system had developed, the plantlets were removed from the vessels, most of the agar was removed from the roots and the plantlets transferred to moist potting soil.

Kanamycin resistant *Nicotiana tabacum* plants were demonstrated to be transformed by PCR analysis following transformation with TOG-1 (SEQ ID NO: 3). Forward primer GUSsense-1:

5'- GGA ATT CAC CGC GTC TTT GAT CGC -3' (SEQ ID NO:16),

and reverse primer nos #2:

5'- GCG CGC GAT AAT TTA TCC -3' (SEQ ID NO:17),

which anneal in the GUS gene and nopaline synthase terminator, respectively, were used to amplify a 513 base pair fragment. Transformation of tobacco plants with plasmids TOP-1 (SEQ ID NO: 4) and TOPI-1 (SEQ ID NO: 5) was also confirmed by PCR using primers NptII-121:

5'- GGG CGC CCG GTT CTT TTT -3' (SEQ ID NO:18),

and NptII- B:

5'- CAG CAA TAT CAC GGG TAG CCA ACG C -3' (SEQ ID NO:19).

Example 13: *Brassica napus* transformation.

Plant transformation vectors were introduced separately into *Agrobacterium tumefaciens* strain GV3101:pMP90 or EHA 101 following the protocol supplied with the Pharmacia *Agrobacterium* cells (product: #27-1535). To prepare the *Agrobacterium* competent cells, 5 ml of YEP medium (10 g yeast extract, 10 g peptone, 5 g sodium chloride per liter, pH 7.0) with 150 μg/ml rifampicin and 100 μg/ml gentamycin was inoculated with a loopful of a glycerol stock of *Agrobacterium tumefaciens* and cultured at 28°C by shaking at 250 rpm approximately 15 h. Two ml of the culture was added to 50 ml of fresh YEP medium and grown at 28°C to an O.D. of 0.5-1.0 at 600 nm. The culture was then chilled on ice for 10 min and centrifuged at 5,000 rpm. The cells were resuspended in 1 ml of cold 20 mM $CaCl_2$. These competent cells were dispensed into pre-chilled 1.5 ml Eppendorf tubes in 100 μl aliquots and frozen at -80°C.

The *Agrobacterium* cells were transformed as follows. One μg of uncut plasmid DNA in water was added to 100 μl of *Agrobacterium* competent cells and incubated on ice for 30 min. The cells were then frozen in liquid nitrogen and thawed quickly at 37°C for 5 min, and 1 ml of YEP medium was added to the cell/DNA mixture and incubated at 28°C for 2 h with gentle shaking (100 rpm). Cells were then centrifuged in a microfuge for 30 s, the supernatant was poured out and the pellet resuspended in the remaining supernatant (50-100 μl). The resuspended cells were spread on a YEP plate with 150 μg/ml rifampicin, 100 μg/ml gentamycin and 50 μg/ml kanamycin, and incubated at 28°C for 2-3 days.

Plasmid DNA from individual *Agrobacterium* colonies was digested and analyzed by agarose gel electrophoresis to verify the integrity of the vector. Colonies which contained the desired recombinant plasmid were selected and grown overnight in 5 ml AB minimal medium with 50 μg/ml kanamycin and 50 μg/ml gentamycin. The overnight culture was centrifuged at 4500 rpm for 15 min and the cells resuspended in 1 ml of double distilled water or 10 mM $MgSO_4$.

Agrobacterium-mediated transformation of *B. napus* cv. Westar was performed according to the method of Moloney et al., *Plant Cell Rep.* **8**:238-242 (1989) with

modifications. Seeds were sterilized by brief wetting in 95% ethanol then 70% commercial bleach (Javex) with a drop of detergent (Tween 20) for 15 min with occasional agitation; 0.025% mercuric chloride with a drop of Tween 20 for 10 min and finally rinsed well with sterile distilled water at least 3 times. Thirty to forty seeds were plated on ½ strength hormone-free MS medium (Sigma) with 1% sucrose in 15X60 mm petri dishes. They were then placed, with the lid removed, into a sterilized Magenta GA-7 vessels and kept at 25°C, with 16 h light/8 h dark and a light intensity of 70-80 μE.

Cotyledons were excised from 4-day old seedlings by gently grasping both petioles just above the point where they join the hypocotyl. The cotyledons were soaked in BASE solution (4.3 g/L MS (GIBCO BRL), 10 ml 100X B5 Vitamins (0.1 g/L nicotinic acid, 1.0 g/L thiamine-HCl, 0.1 g/L pyridoxine-HCl, 10 g/L m-inositol), 2% sucrose, 1 mg/L 2,4-D, pH 5.8; 1% DMSO and 200 μM acetosyringone added after autoclaving) containing *Agrobacterium* cells with the recombinant plant transformation vector. Most of the BASE solution was removed and the cotyledons were incubated at 28°C for 2 days in the dark. The dishes containing the cotyledons were then transferred to 4°C for 3-4 days in the dark. Cotyledons were transferred to plates containing MS B5 selection medium (4.3 g/L MS, 10 ml 100X B5 Vitamins, 3% sucrose, 4 mg/L benzyl adenine (BA) ph 5.8; timentin (300 μg/ml) and kanamycin (20 μg/ml) were added after autoclaving) and left at 25 °C, 16 h light/8 dark with lighting to 70-100 μE. Shoots were transferred to Magenta GA-7 vessels containing MS B5 selection medium without BA. When shoots were sufficiently big they were transferred to Magenta GA-7 vessels containing rooting medium (4.3 g/L MS, 5.0 ml 100X B5 Vitamins, 3% sucrose, 0.1 mg/L α-naphthaleneacetic acid (NAA), pH 5.8; 300 μg/ml timentin and 20 μg/ml kanamycin were added after autoclaving). Once a good root system had developed, the plantlets were removed from the vessels, most of the agar was removed from the roots and the plantlets transferred to moist potting soil.

B. napus plants transformed with plasmid TOG-1 (tapetal oleosin-like *Sta* 41-9/*E. coli* β-glucuronidase (GUS) translational fusion; SEQ ID NO: 3) were assayed histochemically for GUS enzymatic activity in the anther following the methods of Jefferson et al., *Plant Mol. Biol. Rep.* **5**:387-405 (1987) with modifications. Anthers

from developing flower buds were dissected and placed in a 96-well microtitre dish containing 200μl of reaction buffer. The reaction buffer contained 1 mg/ml X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, Sigma), 0.05 M sodium phosphate, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.1% sarcosyl, 0.1% β-mercaptoethanol, 0.5 mM potassium ferricyanide and 0.5 mM potassium ferrocyanide. The staining reaction was carried out overnight at 37 °C. GUS activity was scored as present "+" or absent "-". Using this method, GUS expression was detected in the degenerating tapetum of anthers obtained from 3 mm buds of transformant TOG1b1. GUS activity was detected in tissue obtained from one plant, however, since maximum activity of this promoter occurs during the narrow window of time associated with tapetal degradation this result is not unexpected. Anthers obtained from positive control plant A(+)1 transformed with CaMV 35S/GUS-Int/noster and negative control plant (-)1a exhibited positive and negative GUS activity, respectively. GUS activity is also observed in intact mature pollen.

Table 3

Plant	GUS activity
TOG1a	-
TOG1b1	+
TOG1b2	-
TOG2a	-
TOG2b	-
TOG3a	-
A(+)1	+
(-)1a	-

Example 14: *Brassica carinata* transformation and Western blot analysis of transgenic plants.

Plant transformation vectors were introduced separately into *Agrobacterium tumefaciens* strain GV3101:pMP90 following the protocol supplied with Pharmacia *Agrobacterium* cells (product: #27-1535). To prepare the *Agrobacterium* competent cells, 5 ml of YEP medium (10 g yeast extract, 10 g peptone, 5 g sodium chloride per liter, pH 7.0) with 150 μg/ml rifampicin and 100 μg/ml gentamycin was inoculated with a loopful of a glycerol stock of *Agrobacterium tumefaciens* and cultured at 28°C by shaking at 250 rpm approximately 15 h. Two ml of the culture was added to 50 ml of fresh YEP medium and grown at 28°C to an O.D. of 0.5-1.0 at 600 nm. The culture was then chilled on ice for 10 min and centrifuged at 5,000 rpm. The cells were resuspended in 1 ml of cold 20 mM $CaCl_2$. These competent cells were dispensed into pre-chilled 1.5 ml Eppendorf tubes in 100 μl aliquots and frozen at -80°C.

The *Agrobacterium* cells were transformed as follows. One μg of uncut plasmid DNA in water was added to 100 μl of *Agrobacterium* competent cells and incubated on ice for 30 min. The cells were then frozen in liquid nitrogen and thawed quickly at 37°C for 5 min and 1 ml of YEP medium was added to the cell/DNA mixture and incubated at 28°C for 2 h with gentle shaking (100 rpm). Cells were then centrifuged in a microfuge for 30 s, the supernatant was poured out and the pellet resuspended in the remaining supernatant (50-100 μl). The resuspended cells were spread on a YEP plate with 150 μg/ml rifampicin, 100 μg/ml gentamycin and 50 μg/ml kanamycin, and incubated at 28°C for 2-3 days.

Plasmid DNA from individual *Agrobacterium* colonies was digested and analyzed by agarose gel electrophoresis to verify the integrity of the vector. Individual colonies which contained the desired recombinant plasmid were selected and grown for 2-3 days in 5 ml of LB medium (10 g/L bacto-tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.0) with 50 mg/ml kanamycin with shaking at 28° C. A 50 μl aliquot of this culture was used to inoculate 5 ml of fresh LB medium containing 50 mg/ml kanamycin and incubated as above to an O.D. of 0.1 at 660 nm.

The *B. carinata* seeds were sterilized in 2% PPM (Plant Preservative Mixture, Plant Cell Technology Inc.) for 4 h with gentle stirring and rinsed with 1L of sterilized water. Twenty seeds were plated on fresh seed germination medium (1/2 strength MS pH 5.6 (GIBCO BRL), 1% sucrose and 0,7% phytagar) in a 60 X 20 mm petri dishes fitted inside GA-7 Magenta vessels. They were incubated at 25° C for 3-4 days under a 16 h light/8 h dark photoperiod.

Brassica carinata plants were transformed as described by Babic, M., M.Sc. thesis, Dept. of Crop Science and Plant Ecology, University of Saskatchewan, Saskatoon, Canada (1993) with modifications (Charron F. unpublished results). Healthy green cotyledons were cut at the point where they join the hypocotyl. The petiole of each explant was dipped into the *Agrobacterium* suspension and then transferred to 100 X 25 mm petri dishes with Whatman No. 1 filter paper covering the regeneration medium (MS pH 5.8, 3% sucrose; 2 mg/L BA, 0.05 mg/L NAA and 0.7% phytagar). The explants were incubated at 25° C for 2 days under a 16 h light/8 h dark photoperiod. The explants were then transferred to 100 X 25 mm petri dishes containing the selection medium (MS, 2% sucrose; 2 mg/L BA; 0.05 mg/L NAA; 5 mg/L AgNO3; 500 mg/L soluble PVP-10; 500 mg/L MES pH 5.8 and 0.7% phytagar supplemented with 25 mg/ml kanamycin and 300 mg/ml timentin) and incubated for 2 weeks as above.

Regenerated shoots were transferred to shoot elongation medium (1/2 MS pH 5.8; 2% sucrose; 0.05 mg/L BA; 0.03 mg/L (gibberellic acid) GA 3; 150 mg/L phloroglucinol; 0.9% phytagar supplemented with 25 mg/ml kanamycin and 300 mg/ml timentin) in 60 X 20 mm petri dishes fitted in GA-7 Magenta vessels and incubated for two weeks as above. Shoots were transferred to rooting media (1/2 MS; 1% sucrose; 0.05 mg/L NAA and 0.7% phytagar supplemented with 25 mg/ml kanamycin and 300 mg/ml timentin) and when healthy roots appeared the plantlets were transferred to soil.

Anthers were dissected from *B. carinata* flower buds, frozen in liquid nitrogen and ground to a fine powder. Proteins were extracted from the frozen powder by mixing and sonicating with with 6 μl/mm length of flower bud in 1.2 X SDS PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) loading buffer (75 mM Tris-HCl pH 6.8; 2.4% SDS; 12% glycerol; 6% 2-mercaptoethanol; 0.01% bromophenol blue). The extract was centrifuged for 5 min at 13,000 rpm, the supernatant transferred to a fresh tube and heated to 50° C before loading on the gel.

The SDS PAGE was performed according to the Laemmli, U.K. *Nature* **227**:680-685 (1970) with a 10% acrylamide gel. The gel was blotted electrophoretically (Hoeffer) to a PVDF membrane (Millipore) in 50 mM Tris-HCl, 380 mM glycine, 0.1% SDS and 20% methanol. The membrane was blocked with 5% skim milk powder, 3% bovine serum albumin (BSA) in TBS (50 mM Tris-HCl pH 7.5, 150 mM NaCl) for 1 h at 20°C. The blocked PVDF membrane was incubated with the anti- CPI (cysteine protease inhibitor) antibody diluted 1/12,500 in 0.5% blocking solution (Boehringer Mannnheim). The membrane was washed twice in TBST (TBS + 0.1% Tween 20) for 10 min and twice more in 0.5% blocking solution. The membrane was incubated with the secondary antibody, anti-rabbit horse radish peroxidase conjugate, diluted to 1/2000 in 0.5% blocking solution for 30 min at 20°C. The membrane was then washed 4 times 15 min in TBST at 20°C. Proteins were detected using the Chemiluminescence Blotting Substrate (POD, Boehringer Mannheim) and the membranes were exposed to X-ray film (Kodak, X-Omat).

Western blot analysis was performed on transgenic *Brassica carinata* containing TOPI-1 (SEQ ID NO: 5). The full length *Brassica napus* tapetal oleosin-like *Sta* 41-9/*Onchocerca volvulus* protease inhibitor fusion (expected molecular weight 57 kDa) and the smaller processed version where the N-terminal end of the oleosin-like protein is cleaved (expected molecular weight 47 kDa) were both detected in anther protein extracts from 4 mm flower buds obtained from different transgenic *B. carinata* plants (Figure 20). These proteins were not detected in anther extracts from untransformed *B. carinata* plants. Western blot analysis was also performed on

anther protein extracts from flower buds of transgenic line No. 1 at different stages of development. The higher molecular weight full length 57 kDa fusion protein was detected in anther protein extracts from 3 mm buds and was undetectable in the older 7 mm buds. However, the 47 kDa corresponding to the cleaved protein was evident in the anther extracts from 4 mm buds and persisted through to the late stage of bud development (Figure 21). This reflects the cleavage and targeting to the pollen coat of the native tapetal oleosin-like proteins.

Example 15: Transcriptional fusion of the antisense *Sta* 41-2 tapetal oleosin-like cDNA clone to the *Sta* 41 G(10) promoter.

Plasmid T28 containing the tapetal oleosin-like *Sta* 41 G(10) promoter fragment (Hong, H.P., Ross, J.H.E., Gerster, J.L., Rigas, S., Datla, R.S.S., Hatzopoulos, P., Scoles, G., Keller, W., Murphy, D., Robert, L.S. *Plant Mol. Biol.* **34**:549-555 (1997)) was digested with *Bam* HI and *Hin*d III, and the fragment containing the promoter was cloned into Camter III a derivative of the binary vector Bin 19 (Bevan, M., *Nucleic Acids Res.* **12**:8711-8721 (1984)) containing the nopaline synthase polyadenylation signal. The resulting plasmid was called T1. The tapetal oleosin-like cDNA clone Sta 41-2 was partially digested with *Eco* RI and cloned in the antisense orientation into the *Eco* RI site of T1 to generate the plant transformation vector SAS-1 (Figure 22). SAS-1 was used to transform *Brassica napus* to reduce the levels of tapetal oleosin-like mRNA in the tapetum and consequently cause a reduction of the tapetal oleosin-like protein which is part of the pollen tryphine.

Additional Applications of the Invention

The above examples describe various proteins that can be translationally fused to tapetal oleosin-like proteins, pollen or stigma proteins, and targeted to the surface of pollen or stigma. One skilled in the art can readily modify the above techniques to produce transgenic plants containing any desired protein or

polypeptide on the surface of pollen or stigma. Different polypeptides or proteins that can be used according to the present invention are provided but not limited to the examples below.

The present invention has been described with regard to preferred embodiments. However, it will be obvious to persons skilled in the art that a number of variations and modifications can be made without departing from the scope of the invention as described herein

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OF PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A method for modifying the extracellular compartment of a floral cell of a plant, said method comprising:

expressing a construct comprising a gene of interest within an anther or pistil cell, said gene of interest encoding a protein, fusion protein or peptide, or a fragment of said protein, fusion protein or peptide, said protein, fusion protein or peptide, or a fragment of said protein, fusion protein or peptide capable of modifying the composition of the extracellular compartment of said floral cell and altering either the function, use, or development of said floral cell, or modifying the interaction of said floral cell with other cells.

2. The method of claim 1 wherein said gene of interest is native to said plant.

3. The method of claim 1 wherein said gene of interest is non-native to said plant.

4. The method of claim 1 wherein said construct is a chimeric gene construct.

5. The method of claim 1 wherein said floral cell is a pollen grain, and said protein, fusion protein or peptide, or a fragment of said protein, fusion protein or peptide is released into a locule of an anther thereby associating with said extracellular compartment of said pollen grain.

6. The method of claim 1 wherein said floral cell is either a pollen grain or a stigma cell, and said construct comprises a translated sequence capable of directing the extracellular localization of said protein, fusion protein or peptide, or a fragment of said protein, fusion protein or peptide on said floral cell.

7. The method of claim 6, wherein said translated sequence is selected from the group consisting of a signal peptide, a hydrophobic domain, or a combination thereof.

8. The method of claim 6 wherein said translated sequence is a protein, or fragment thereof, known to be targeted to the extracellular compartment of a floral cell.

9. The method of claim 8 wherein said protein or fragment thereof is an oleosin-like protein.

10. The method of claim 8 wherein said protein or fragment thereof is selected from the group consisting of Sta 41-2 or Sta 41-9, Sta 44, SLG_{WS1} or GPis363

11. A method for obtaining the localization of a protein, fusion protein, or peptide of interest, or a fragment of the protein, fusion protein, or peptide, within or on the extracellular compartment of a floral cell, comprising:

i) preparing a construct comprising:
   a) a promoter sequence capable of expressing a gene encoding said protein, fusion protein, or peptide of interest, or a fragment of the protein, fusion protein, or peptide, within the floral cell;
   b) a gene that encodes said protein, fusion protein, or peptide of interest, or a fragment of the protein, fusion protein, or peptide; and
   c) a translated sequence capable of directing the extracellular localization of said protein, fusion protein, or peptide of interest, or a fragment of the protein, fusion protein, or peptide, on the floral cell;
   d) a terminator sequence; and
ii) transforming a plant with said construct;

wherein the floral cell, is selected from the group consisting of pollen, anther or pistil cells.

12. The method of claim 11, wherein the translated sequence of step c) is selected from the group consisting of a signal peptide, a hydrophobic domain, or a combination thereof.

13. The method of claim 11, wherein the translated sequence of step c) is a protein, or fragment thereof, known to be targeted to the extracellular compartment of a floral cell.

14. The method of claim 13 wherein said protein or fragment thereof is an oleosin-like protein.

15. The method of claim 13 wherein said protein or fragment thereof is selected from the group consisting of Sta 41-2 or Sta 41-9, Sta 44, SLG_{WS1} or GPis363.

16. A method of chemically linking a protein or peptide of interest to the pollen coat comprising:

a) activating pollen grains with a desired reagent for conjugation;

b) adding the protein of interest

17. A microspore or a pollen, or combination thereof prepared by the method of claim 16.

18. A microspore or a pollen, or combination thereof, prepared using the method of claim 1.

19. A vector comprising:

a) a promoter sequence capable of expressing a gene encoding a protein, fusion protein, or peptide of interest, or a fragment of the protein, fusion protein, or peptide, within a floral cell;

b) a gene that encodes the protein, fusion protein, or peptide of interest, or a fragment of the protein, fusion protein, or peptide;

c) a translated sequence capable of directing the extracellular localization of the protein, fusion protein, or peptide of interest, or a fragment of the protein, fusion protein, or peptide, on the floral cell; and

d) a terminator sequence.

20. A transgenic plant cell comprising the vector of claim 19.

21. A transgenic plant comprising the vector of claim 19

22. A seed obtained from the transgenic plant of claim 19.

23. A pollen characterized in that the extracellular compartment of the pollen is modified, said extracellular compartment comprising a protein, a fusion protein, or a peptide of interest, or a fragment of the protein, fusion protein, or peptide.

24. A transgenic plant comprising the pollen as claimed in claim 23.

25. A seed obtained from the transgenic plant of claim 24

26. The method of claim 1, wherein the floral cell is a pollen, and the extracellular compartment comprises either the tryphine, exine, nexine, sexine, or intine, or a combination thereof.

27. The method of claim 1, wherein the floral cell is a pistil cell, and the extracellular compartment comprises the cuticle, cell wall, pellicle, transmitting tract, or extracellular secretions, or combination thereof.

28. A method of modifying pollen-pistil interaction or function comprising, producing a microspore, pollen, or pistil cell, or combination thereof, within a

plant using the method of claim 1, so that the microspore, pollen, or pistil cell, or combination thereof, comprises a modified extracellular domain.

29. The method of claim 28 wherein the extracellular protein, protein fusion or peptide of interest is localized to the microspore, or pollen, or combination thereof.

30. The method of claim 28 wherein the extracellular protein, protein fusion or peptide of interest is localized to the pistil cell.

31 The method of claim 28, wherein the pollen-pistil interaction or function being modified mediates, produces or prevents self compatibility, self incompatibility, out-crossing, in-crossing or a combination thereof.

32. A pistil cell prepared using the method of claim 1.

33. A transgenic plant comprising the pistil cell as claimed in claim 32.

34. A seed obtained from the transgenic plant of claim 33.

35. A stigma cell characterized in that the extracellular compartment of said cell is modified, said extracellular compartment comprising a protein, fusion protein, or peptide of interest, or a fragment of the protein, fusion protein, or peptide, encoded by a chimeric gene construct.

36. A transgenic plant comprising the pistil cell as claimed in claim 35.

37. A seed obtained from the transgenic plant of claim 36.

38. The method of claim 1 wherein the protein, fusion protein, or peptide of interest, or the fragment of a protein, fusion protein, or peptide is localized on the surface of pollen for the purpose of peptide display.

39. The method of claim 1 wherein the protein, fusion protein, or peptide of interest, or a fragment of the protein, fusion protein, or peptide, is localized on the surface of pollen and it is an antibody or antigen.

40. The method of claim 1 wherein the protein, fusion protein, or peptide of interest, or a fragment of the protein, fusion protein, or peptide, is localized on the surface of the pollen grain and it is effective in controlling insect growth, behaviour, feeding, development, or reproduction, or a combination thereof.

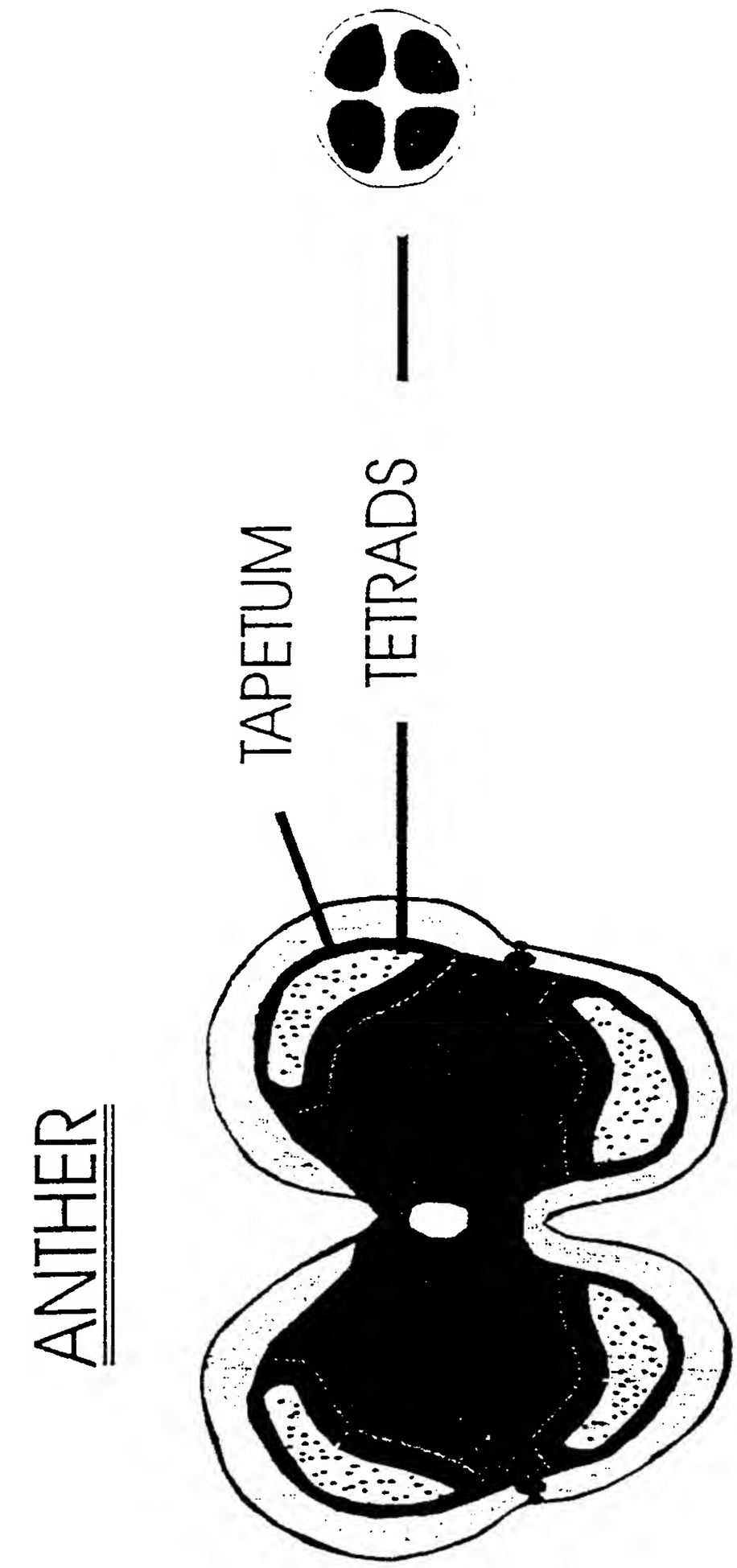


FIG. 1A

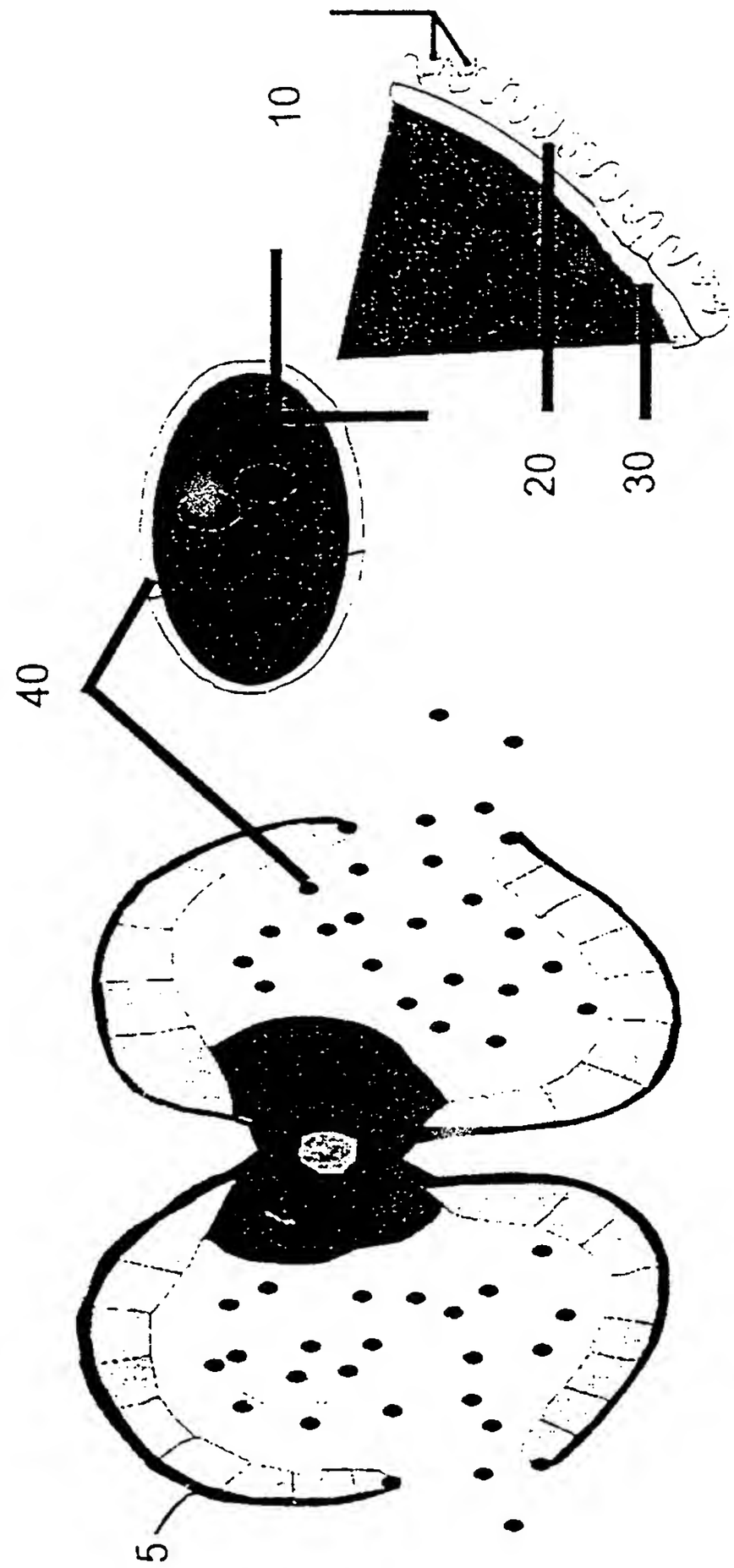


FIG. 1B

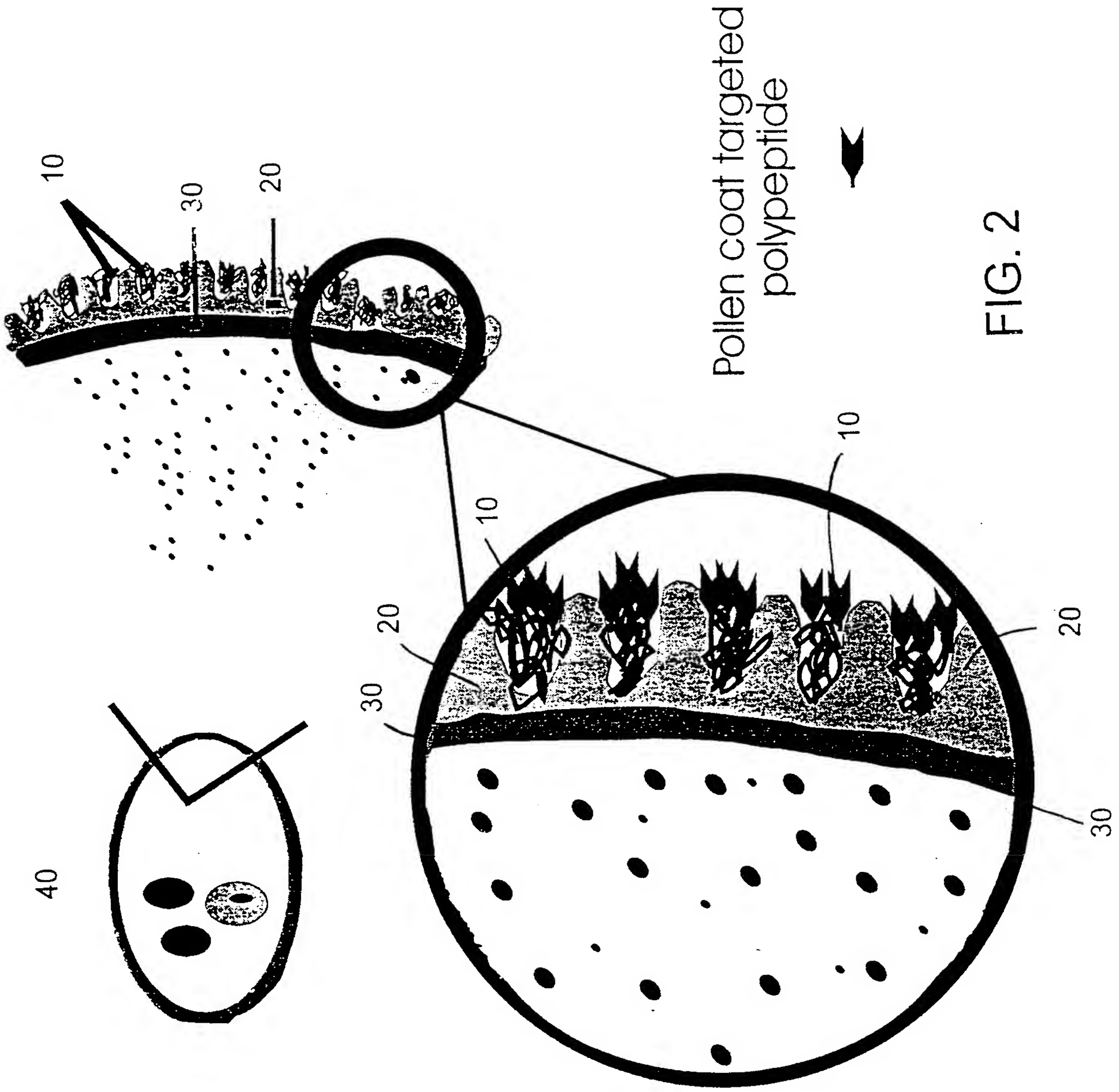
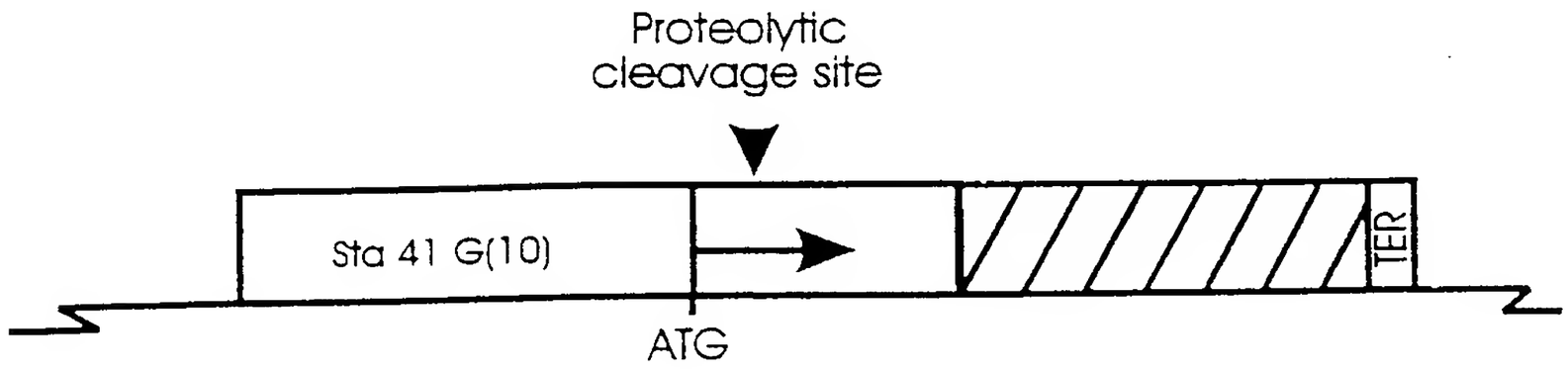
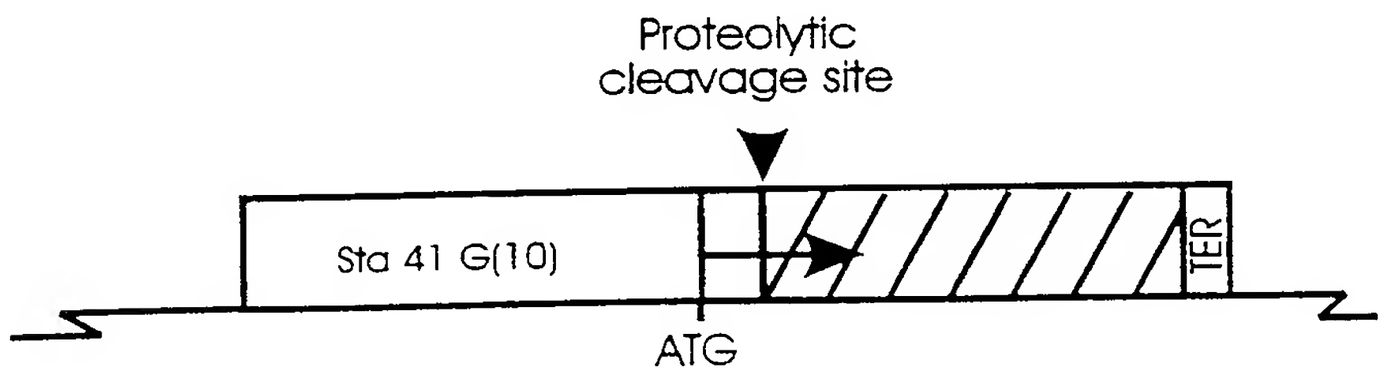


FIG. 2

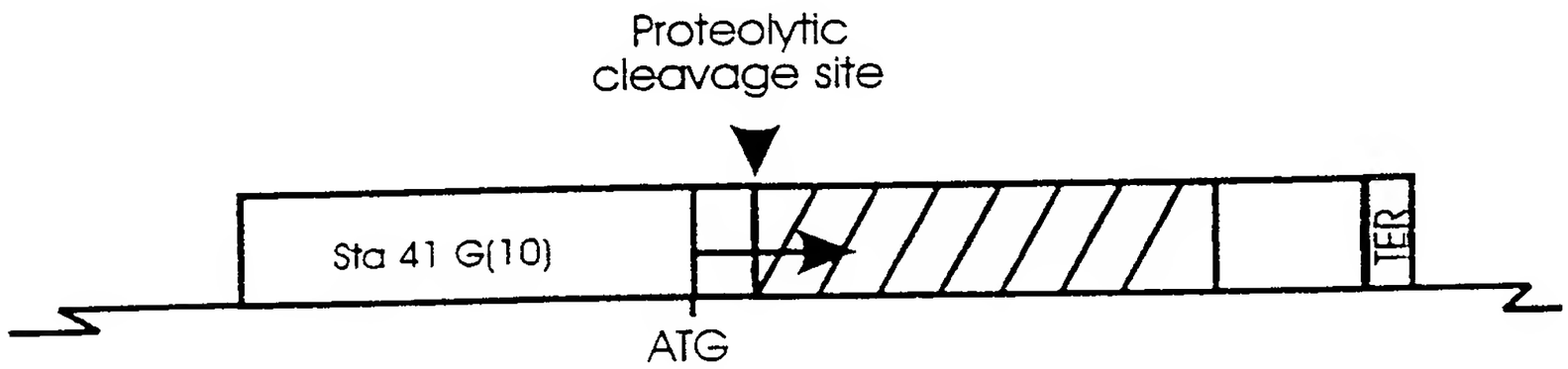
(Fig. 3A) C-terminal translational fusion with full length tapetal oleosin



(Fig. 3B) C-terminal translational fusion at proteolytic cleavage site



(Fig. 3C) Internal translational fusion at proteolytic cleavage site



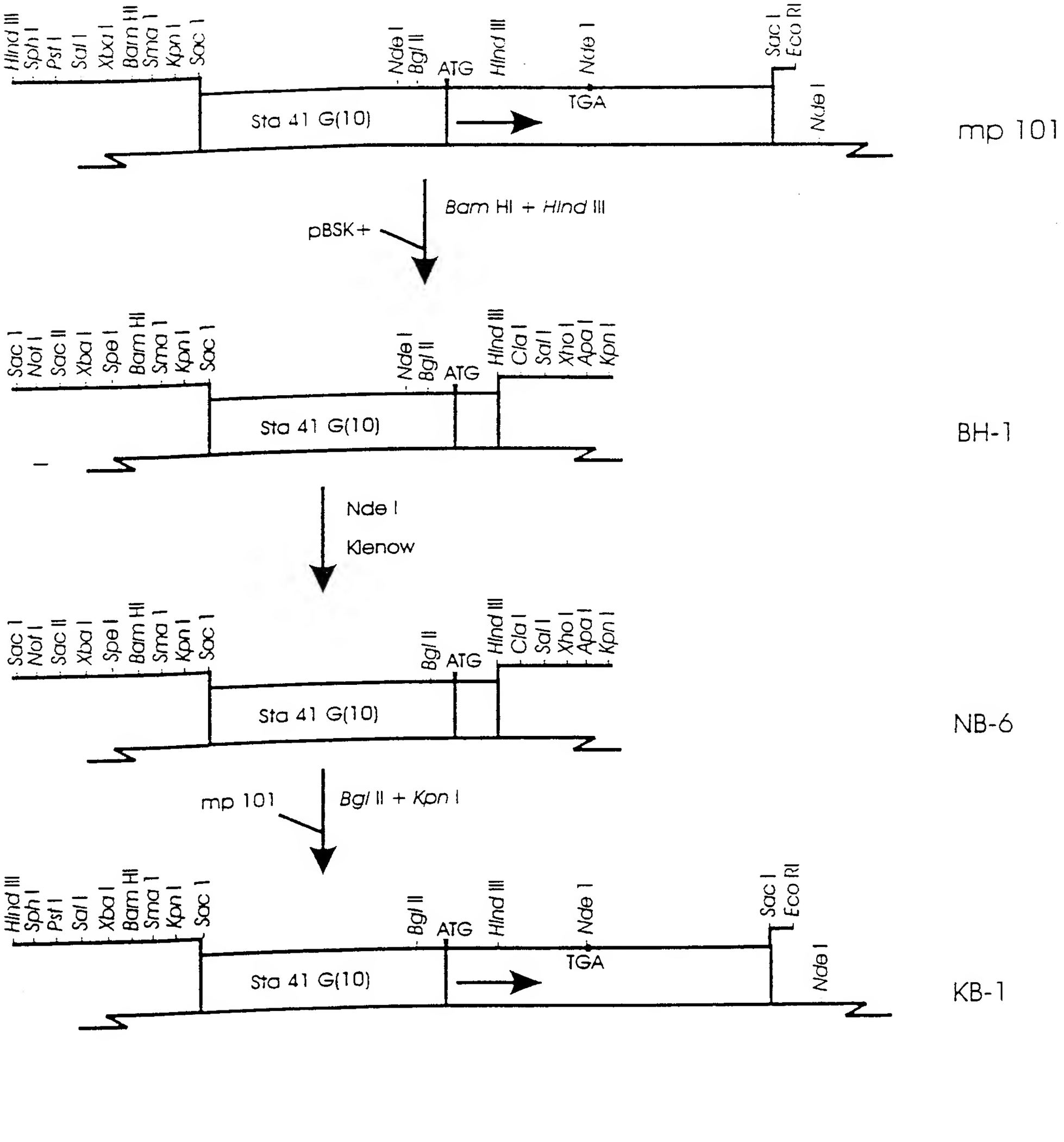

Hind III
Sph I
Pst I
Sal I
Xba I
Bam HI
Sma I
Kpn I
Sac I
Nde I
Bgl II
ATG
Hind III
Nde I
Sac I
Eco RI
Nde I
Sta 41 G(10)
TGA
mp 101
Bam HI + Hind III
pBSK+
Sac I
Not I
Sac II
Xba I
Spe I
Bam HI
Sma I
Kpn I
Sac I
Nde I
Bgl II
ATG
Hind III
Cla I
Sal I
Xho I
Apa I
Kpn I
Sta 41 G(10)
BH-1
Nde I
Klenow
Sac I
Not I
Sac II
Xba I
Spe I
Bam HI
Sma I
Kpn I
Sac I
Bgl II
ATG
Hind III
Cla I
Sal I
Xho I
Apa I
Kpn I
Sta 41 G(10)
NB-6
mp 101
Bgl II + Kpn I
Hind III
Sph I
Pst I
Sal I
Xba I
Bam HI
Sma I
Kpn I
Sac I
Bgl II
ATG
Hind III
Nde I
Sac I
Eco RI
Nde I
Sta 41 G(10)
TGA
KB-1

FIG. 4A

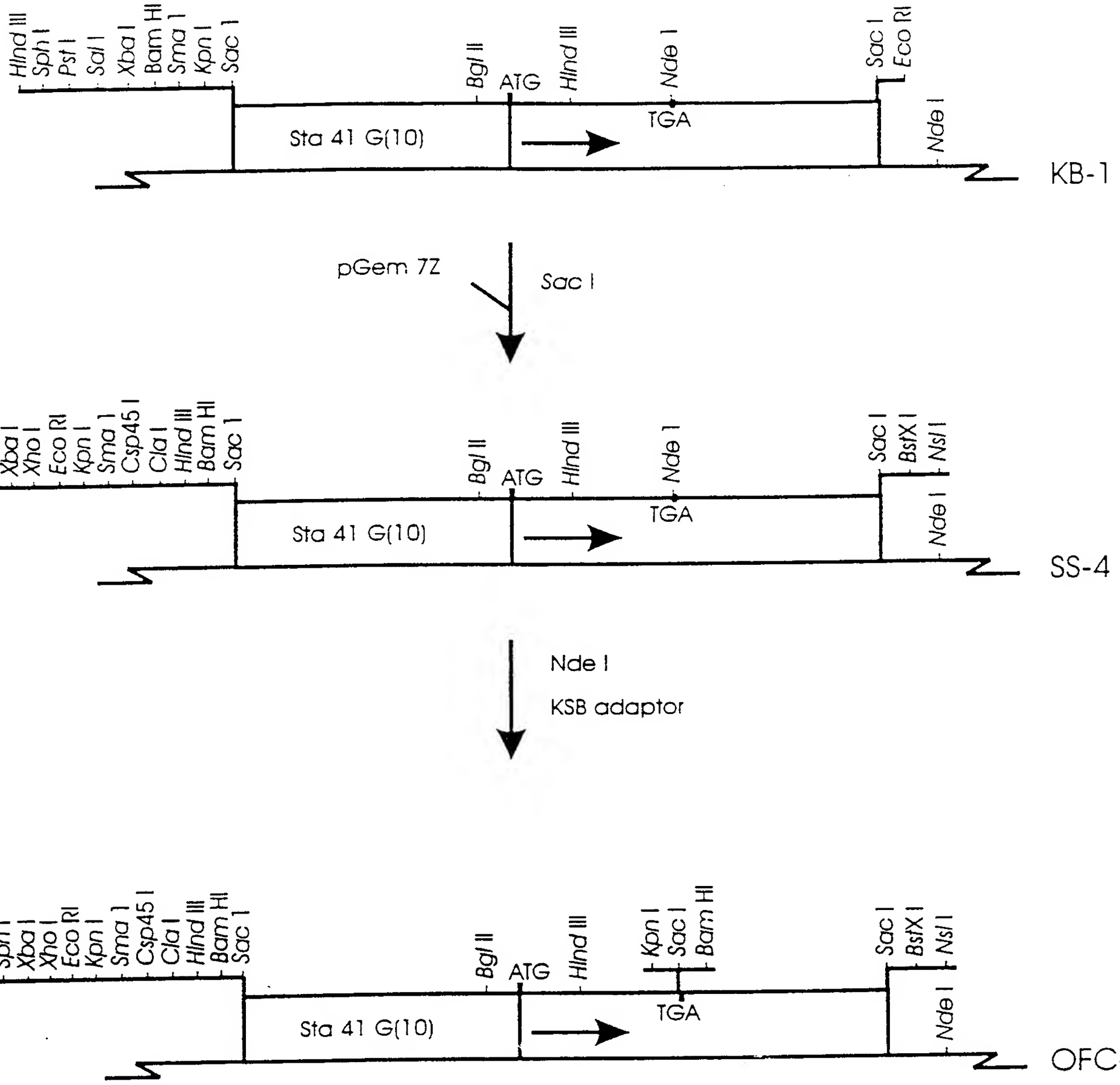
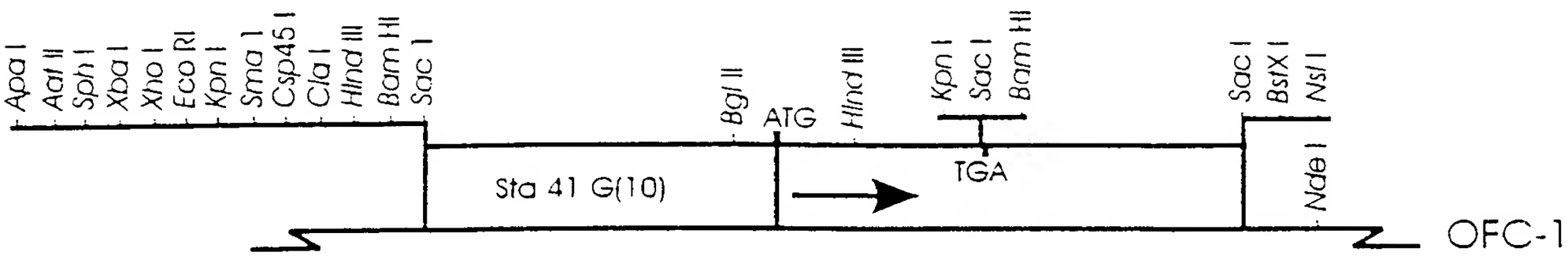
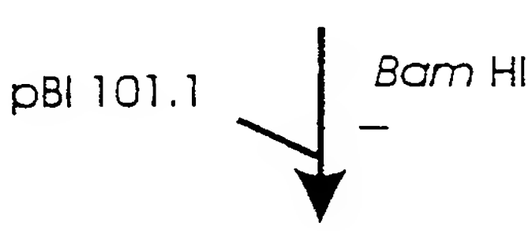

Hind III
Sph I
Pst I
Sal I
Xba I
Bam HI
Sma I
Kpn I
Sac I
Bgl II
ATG
Hind III
Nde I
TGA
Sac I
Eco RI
Nde I
Sta 41 G(10)
KB-1
pGem 7Z
Sac I
Apa I
Aat II
Sph I
Xba I
Xho I
Eco RI
Kpn I
Sma I
Csp45 I
Cla I
Hind III
Bam HI
Sac I
Bgl II
ATG
Hind III
Nde I
TGA
Sac I
BstX I
Nsi I
Nde I
Sta 41 G(10)
SS-4
Nde I
KSB adaptor
Apa I
Aat II
Sph I
Xba I
Xho I
Eco RI
Kpn I
Sma I
Csp45 I
Cla I
Hind III
Bam HI
Sac I
Bgl II
ATG
Hind III
Kpn I
Sac I
Bam HI
TGA
Sac I
BstX I
Nsi I
Nde I
Sta 41 G(10)
OFC-1

FIG. 4B


Apa I
Aat II
Sph I
Xba I
Xho I
Eco RI
Kpn I
Sma I
Csp45 I
Cla I
Hind III
Bam HI
Sac I
Bgl II
ATG
Hind III
Kpn I
Sac I
Bam HI
Sac I
BstX I
Nsi I
Nde I
Sta 41 G(10)
TGA
OFC-1


pBI 101.1
Bam HI

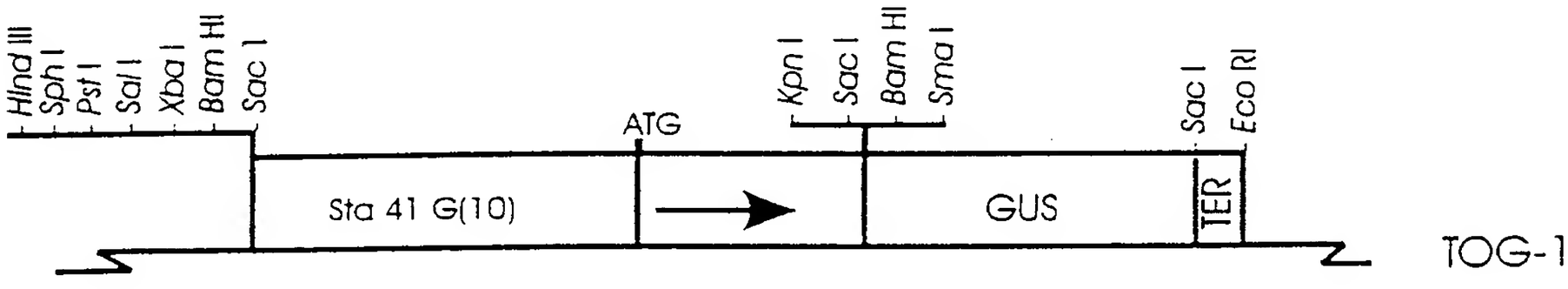

Hind III
Sph I
Pst I
Sal I
Xba I
Bam HI
Sac I
ATG
Kpn I
Sac I
Bam HI
Sma I
Sac I
Eco RI
Sta 41 G(10)
GUS
TER
TOG-1

FIG. 5

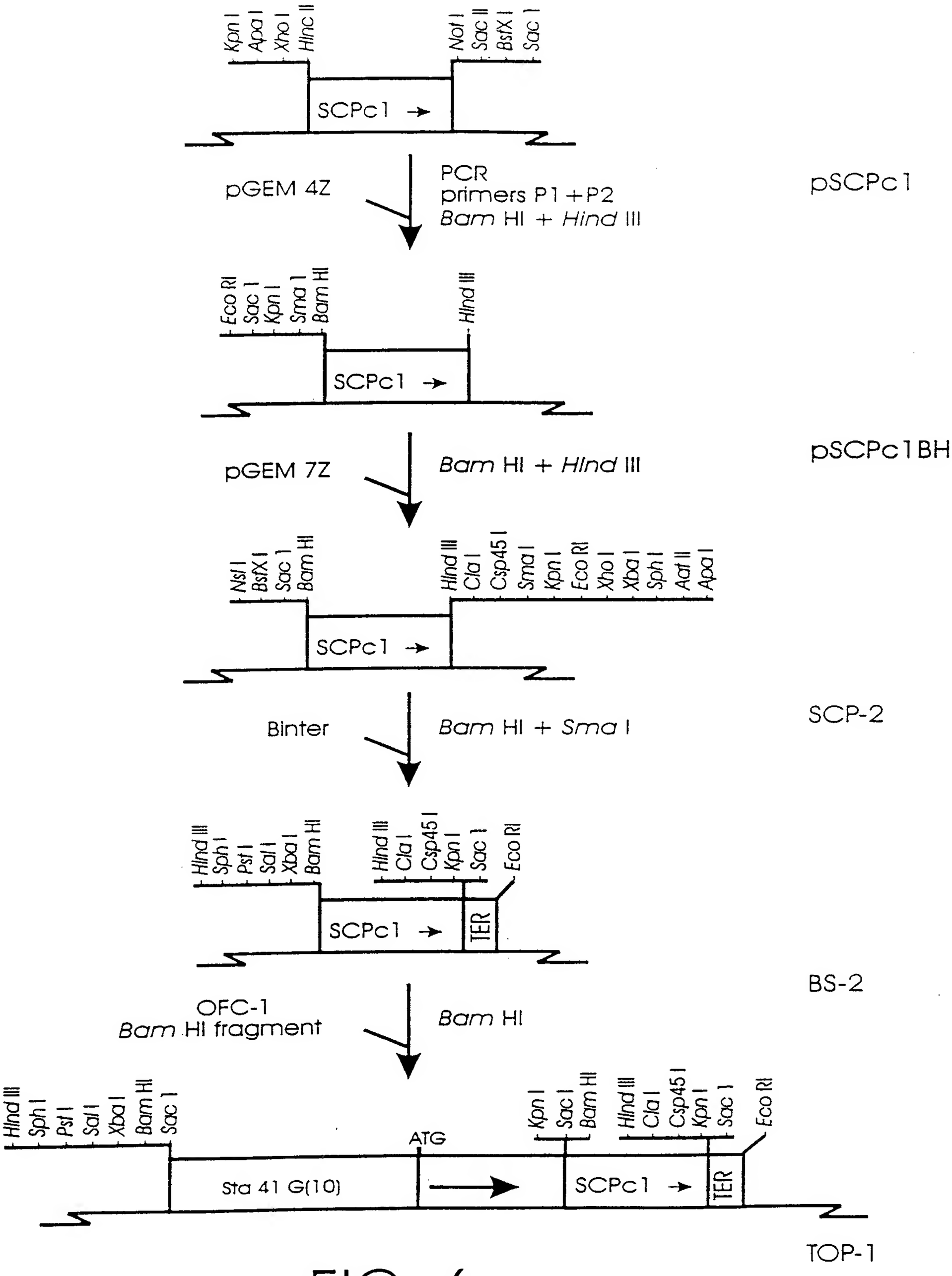

Kpn I
Apa I
Xho I
Hinc II
Not I
Sac II
BstX I
Sac I
SCPc1
pGEM 4Z
PCR
primers P1 + P2
Bam HI + Hind III
pSCPc1
Eco RI
Sac I
Kpn I
Sma I
Bam HI
Hind III
SCPc1
pGEM 7Z
Bam HI + Hind III
pSCPc1BH
Nsi I
BstX I
Sac I
Bam HI
Hind III
Cla I
Csp45 I
Sma I
Kpn I
Eco RI
Xho I
Xba I
Sph I
Aat II
Apa I
SCPc1
Binter
Bam HI + Sma I
SCP-2
Hind III
Sph I
Pst I
Sal I
Xba I
Bam HI
Hind III
Cla I
Csp45 I
Kpn I
Sac I
Eco RI
SCPc1
TER
OFC-1
Bam HI fragment
Bam HI
BS-2
Hind III
Sph I
Pst I
Sal I
Xba I
Bam HI
Sac I
ATG
Kpn I
Sac I
Bam HI
Hind III
Cla I
Csp45 I
Kpn I
Sac I
Eco RI
Sta 41 G(10)
SCPc1
TER
TOP-1

FIG. 6

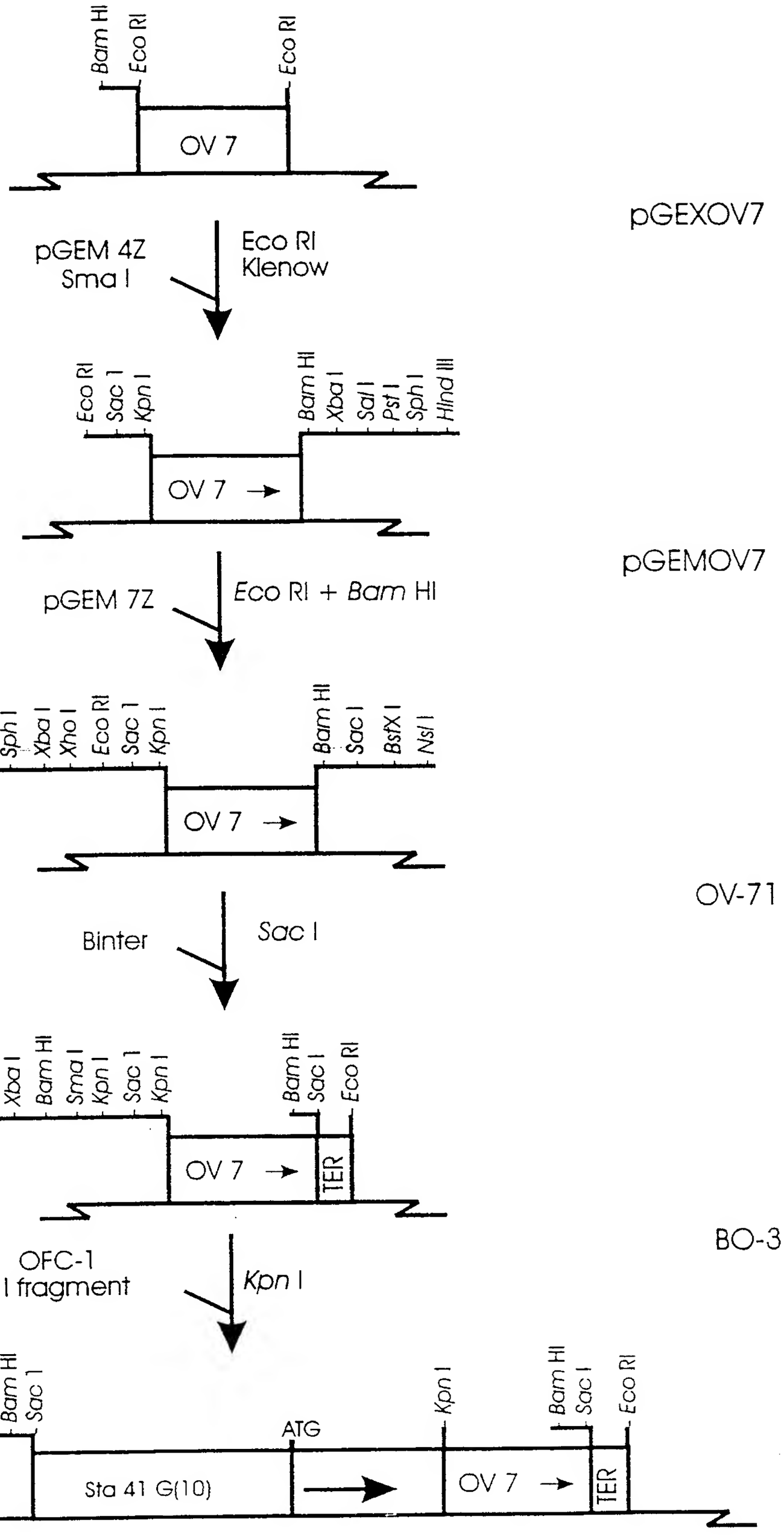
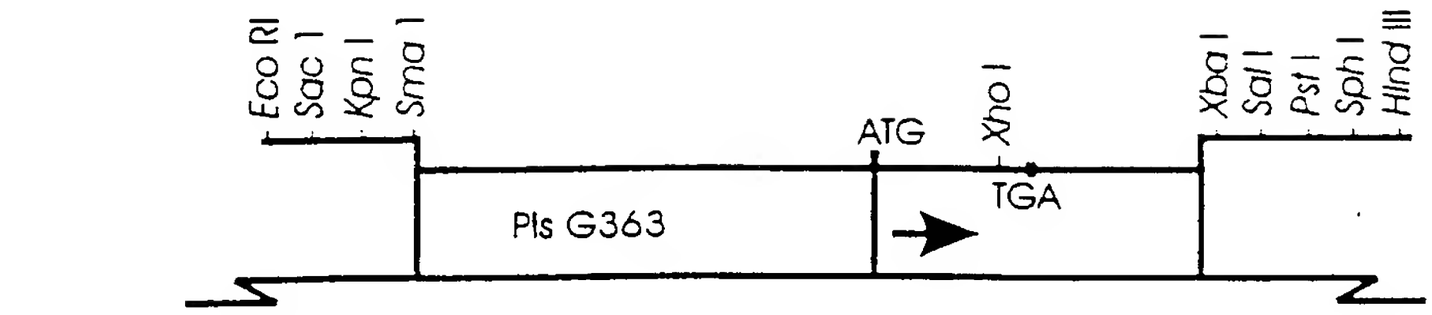

Bam HI
Eco RI
Eco RI
OV 7
pGEXOV7
pGEM 4Z
Sma I
Eco RI
Klenow
Eco RI
Sac I
Kpn I
Bam HI
Xba I
Sal I
Pst I
Sph I
Hind III
OV 7
pGEMOV7
pGEM 7Z
Eco RI + Bam HI
Apa I
Aat II
Sph I
Xba I
Xho I
Eco RI
Sac I
Kpn I
Bam HI
Sac I
BstX I
Nsi I
OV 7
OV-71
Binter
Sac I
Hind III
Sph I
Pst I
Sal I
Xba I
Bam HI
Sma I
Kpn I
Sac I
Kpn I
Bam HI
Sac I
Eco RI
OV 7
TER
BO-3
OFC-1
Kpn I fragment
Kpn I
Hind III
Sph I
Pst I
Sal I
Xba I
Bam HI
Sma I
Kpn I
Sma I
Csp45 I
Cla I
Hind III
Bam HI
Sac I
ATG
Kpn I
Bam HI
Sac I
Eco RI
Sta 41 G(10)
OV 7
TER
TOPI-1

FIG. 7


Eco RI
Sac I
Kpn I
Sma I
ATG
Xho I
Xba I
Sal I
Pst I
Sph I
Hind III
TGA
Pls G363

Bg 2

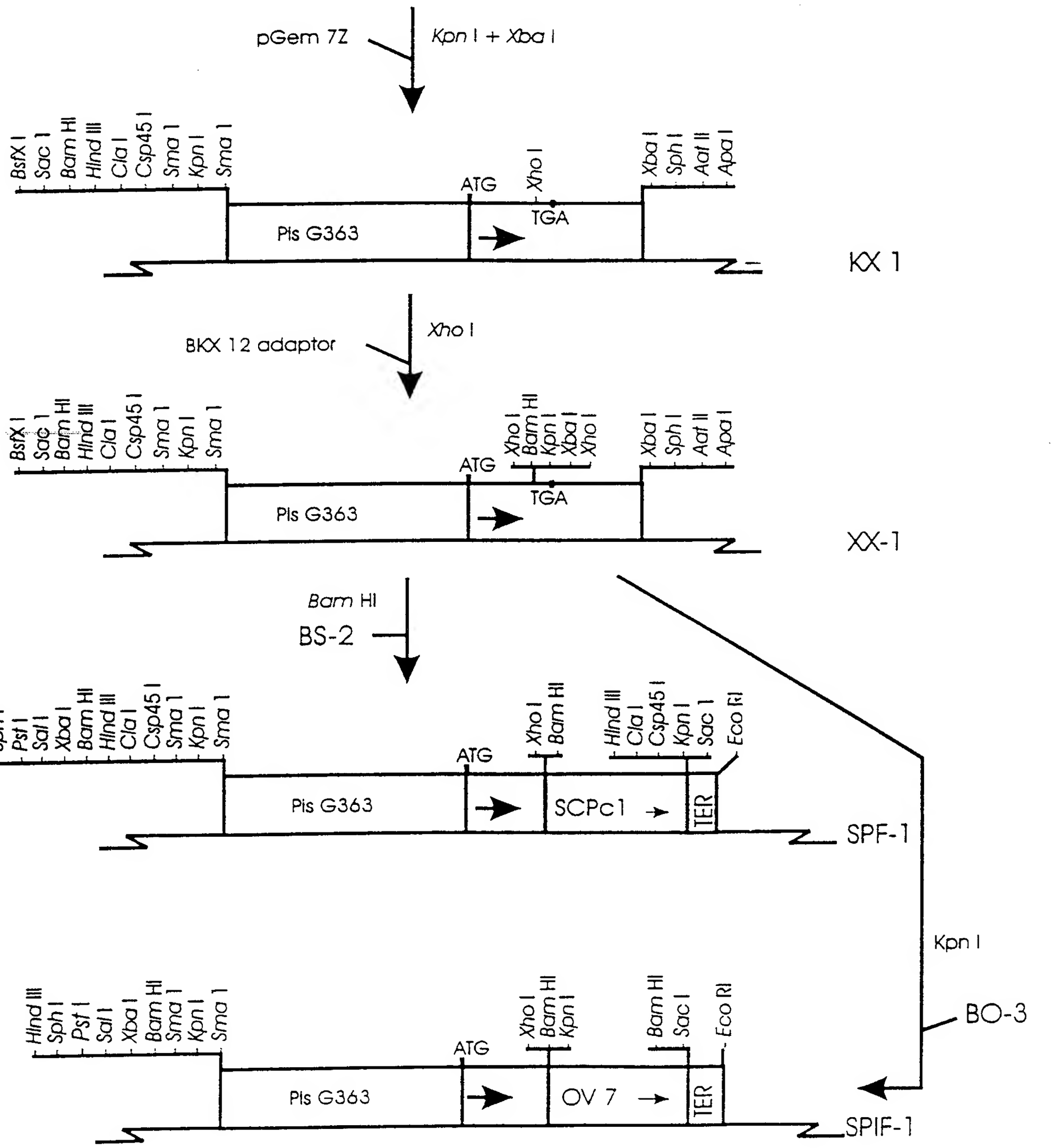

pGem 7Z
Kpn I + Xba I
BsfX I
Sac I
Bam HI
Hind III
Cla I
Csp45 I
Sma I
Kpn I
Sma I
ATG
Xho I
Xba I
Sph I
Aat II
Apa I
TGA
Pls G363
KX 1
BKX 12 adaptor
Xho I
Xho I
Bam HI
Kpn I
Xba I
Xho I
XX-1
Bam HI
BS-2
Hind III
Sph I
Pst I
Sal I
Xba I
Bam HI
Hind III
Cla I
Csp45 I
Sma I
Kpn I
Sma I
Xho I
Bam HI
Hind III
Cla I
Csp45 I
Kpn I
Sac I
Eco RI
SCPc1
TER
SPF-1
Kpn I
BO-3
Xho I
Bam HI
Kpn I
Bam HI
Sac I
Eco RI
OV 7
SPIF-1

FIG. 8

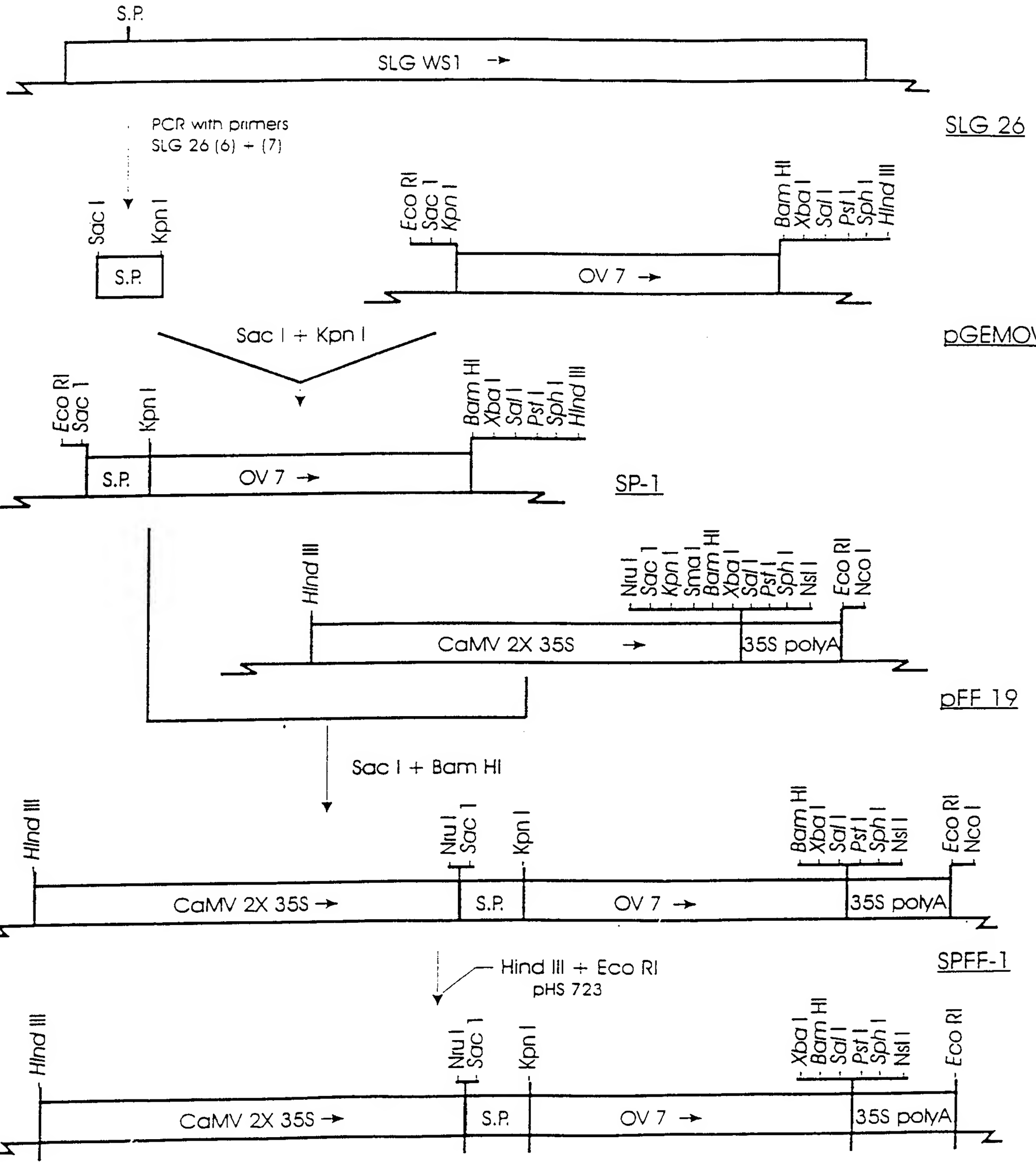
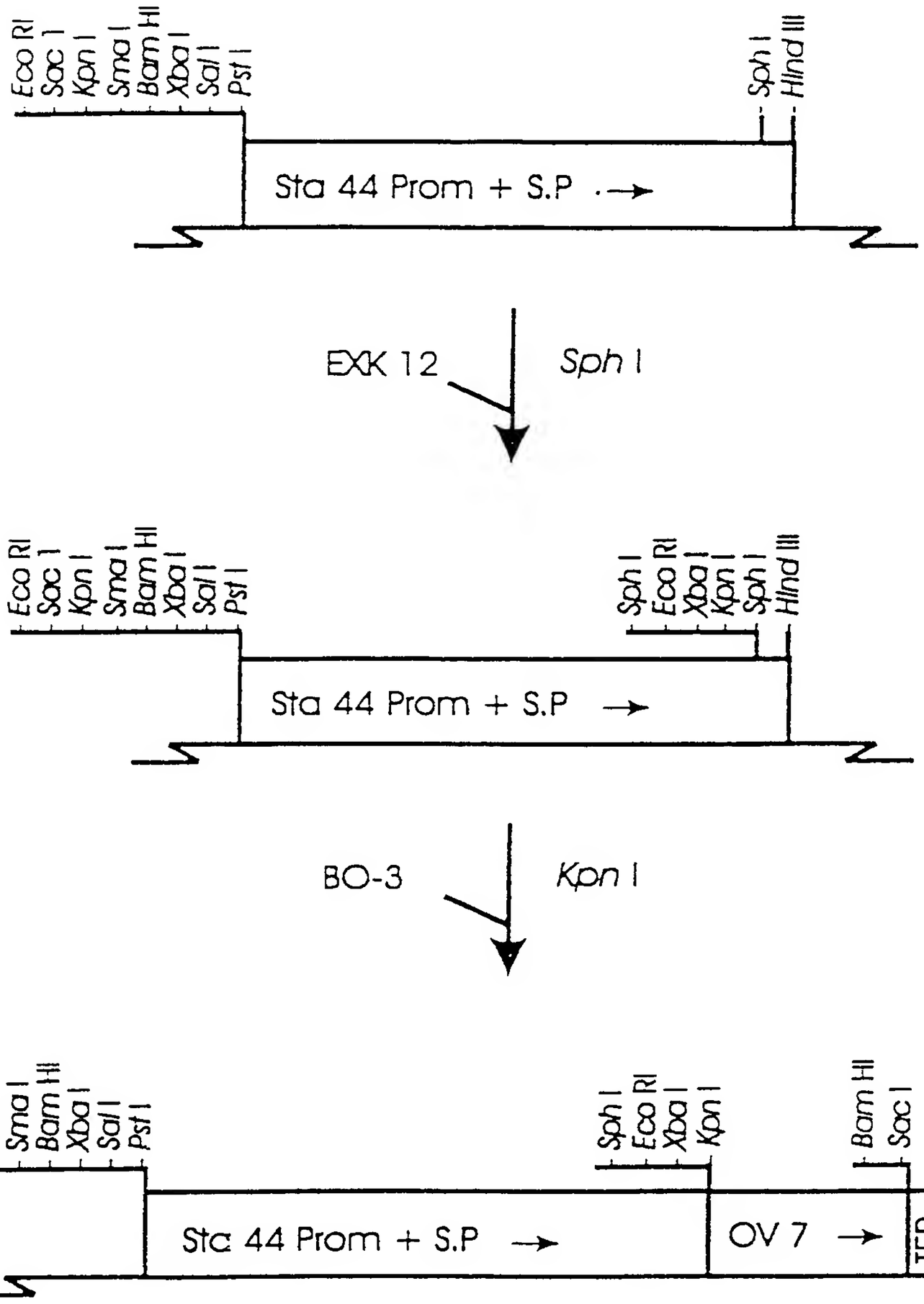

S.P.
SLG WS1
SLG 26
PCR with primers
SLG 26 (6) + (7)
Sac I
Kpn I
S.P.
Eco RI
Sac I
Kpn I
Bam HI
Xba I
Sal I
Pst I
Sph I
Hind III
OV 7
pGEMOV7
Sac I + Kpn I
SP-1
Hind III
Nru I
Sac I
Kpn I
Sma I
Bam HI
Xba I
Sal I
Pst I
Sph I
Nsi I
Eco RI
Nco I
CaMV 2X 35S
35S polyA
pFF 19
Sac I + Bam HI
SPFF-1
Hind III + Eco RI
pHS 723
SPOV 1

FIG.9



HP-9

SS-1

POV-1

FIG. 10

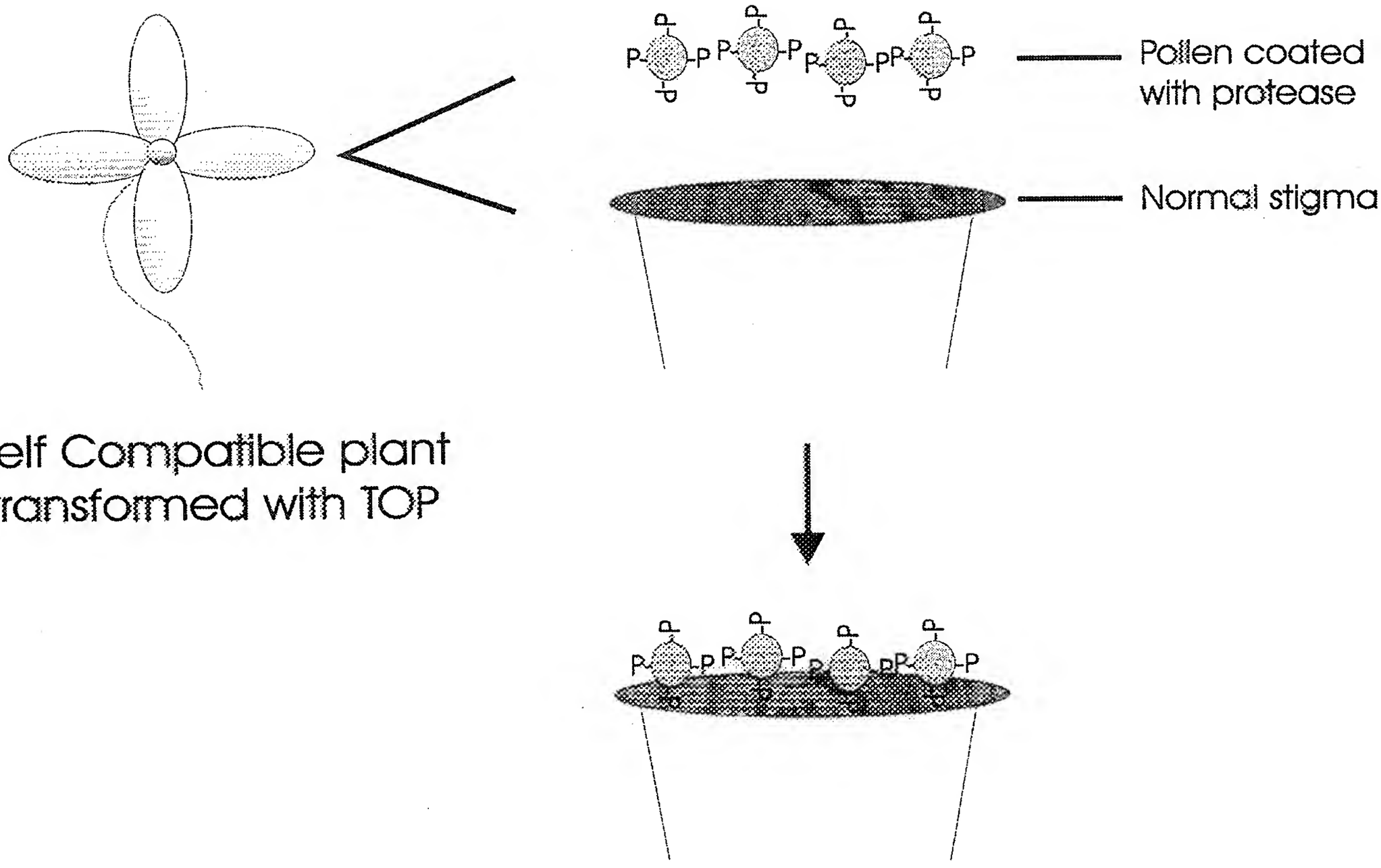

Pollen coated with protease
Normal stigma
Self Compatible plant transformed with TOP
- No self-fertilization
- No outcrossing
- Pollen can only germinate on stigmas expressing PI
- Incrossing possible

FIG.11

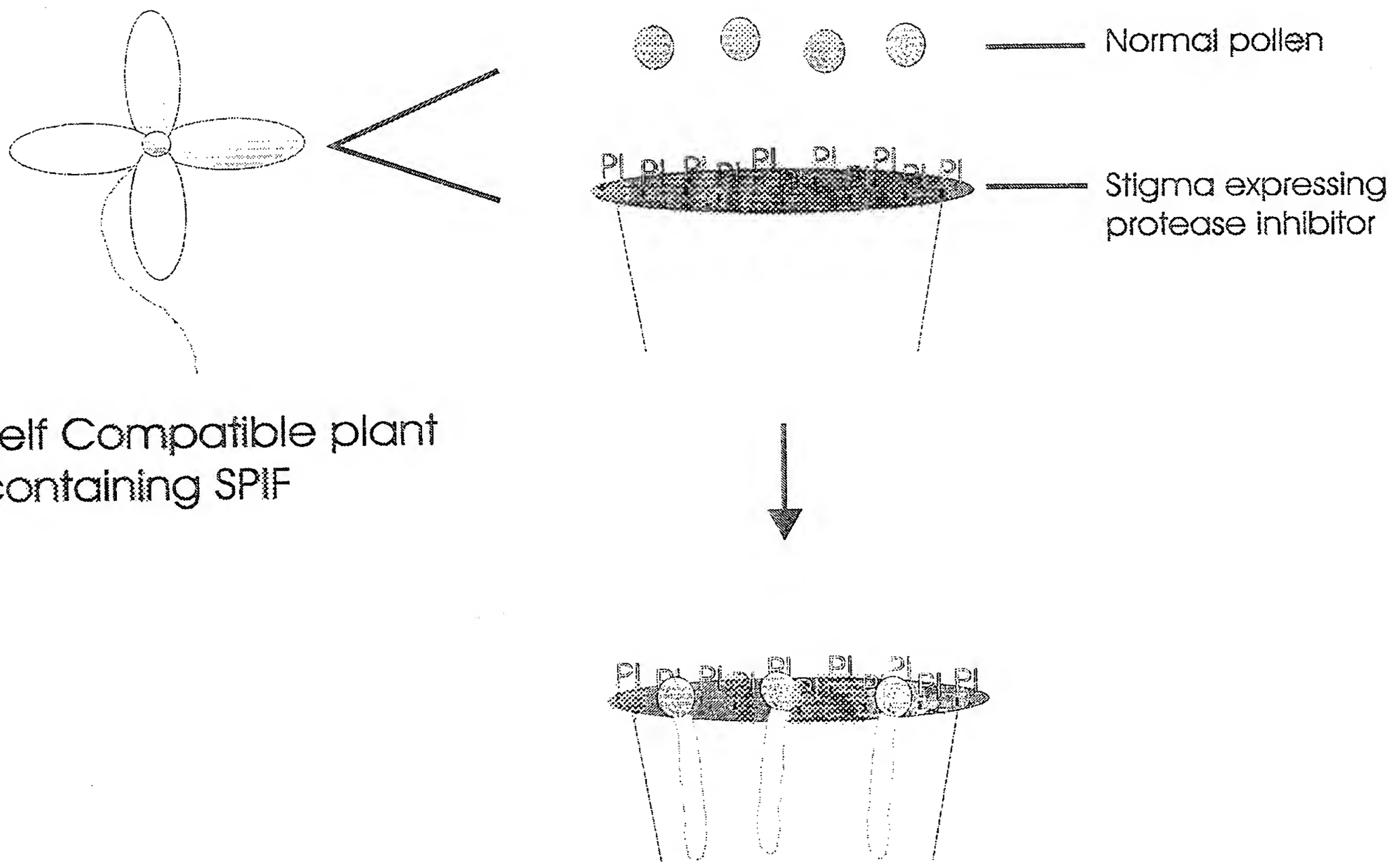

Normal pollen
Stigma expressing protease inhibitor
Self Compatible plant containing SPIF
- Self-fertilization occurs
- Pollen expressing protease can also germinate
- Outcrossing possible

FIG. 12

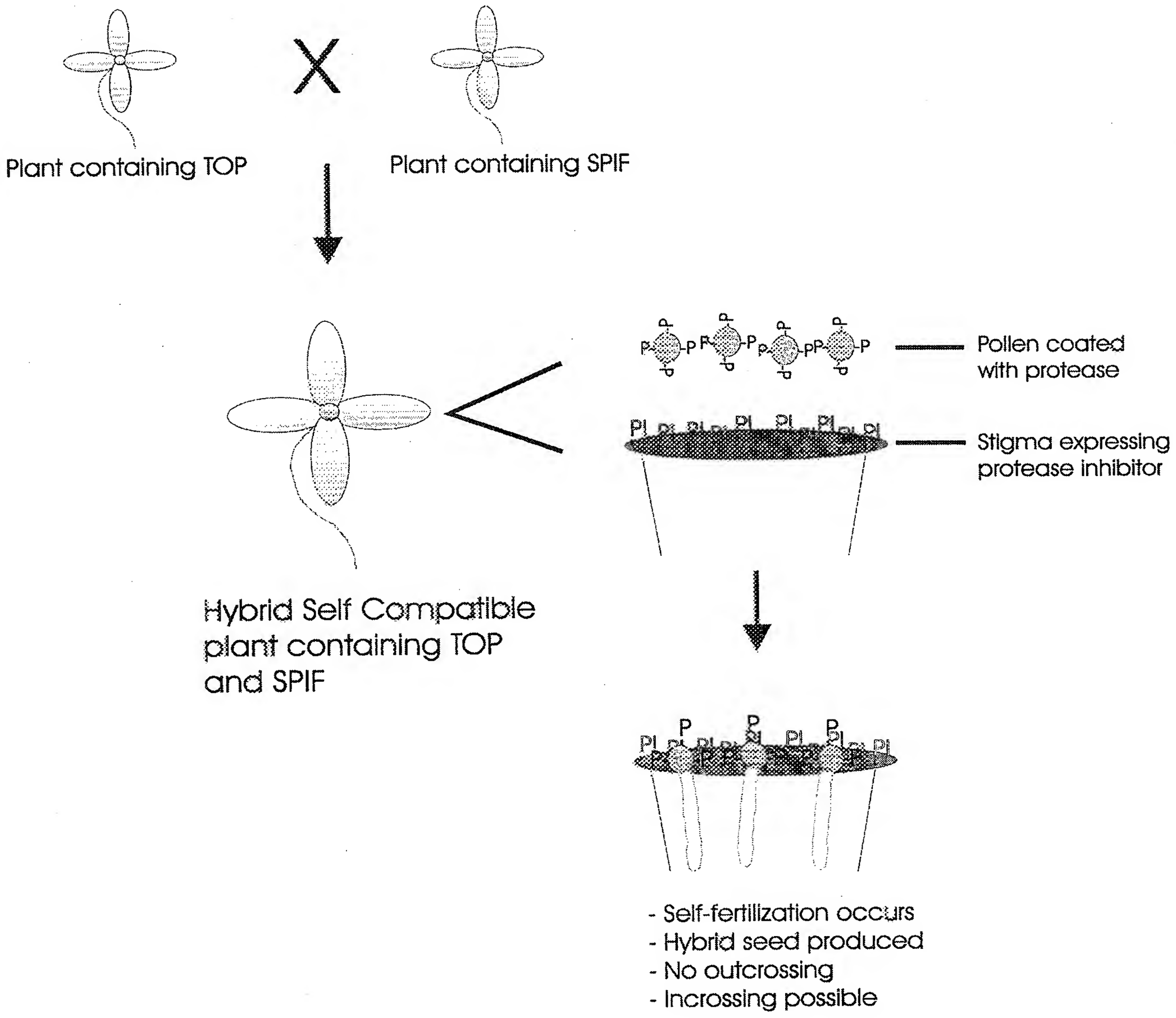

Plant containing TOP
X
Plant containing SPIF
Hybrid Self Compatible plant containing TOP and SPIF
Pollen coated with protease
Stigma expressing protease inhibitor
- Self-fertilization occurs
- Hybrid seed produced
- No outcrossing
- Incrossing possible

FIG. 13

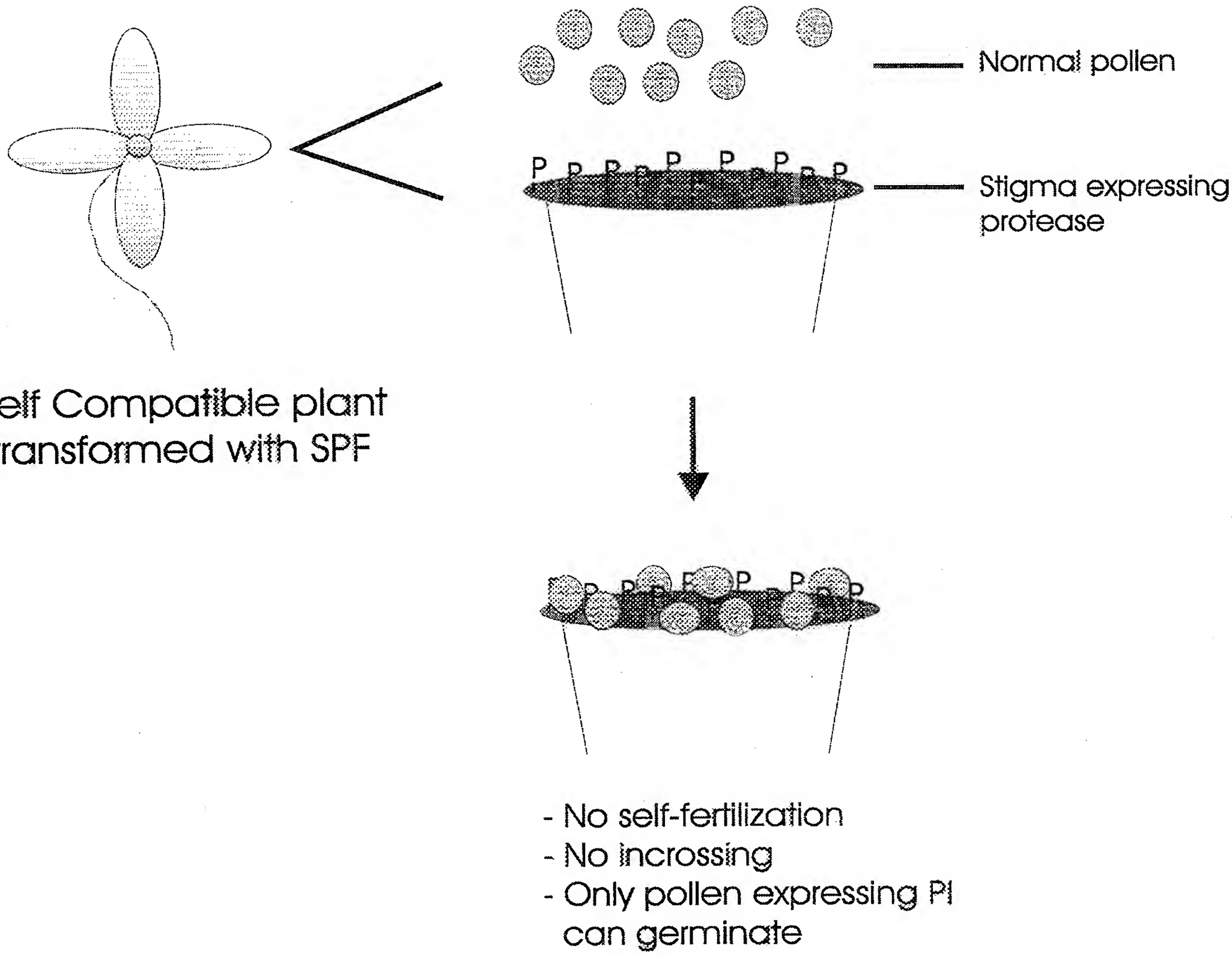

Normal pollen
Stigma expressing protease
Self Compatible plant transformed with SPF
- No self-fertilization
- No incrossing
- Only pollen expressing PI can germinate

FIG. 14

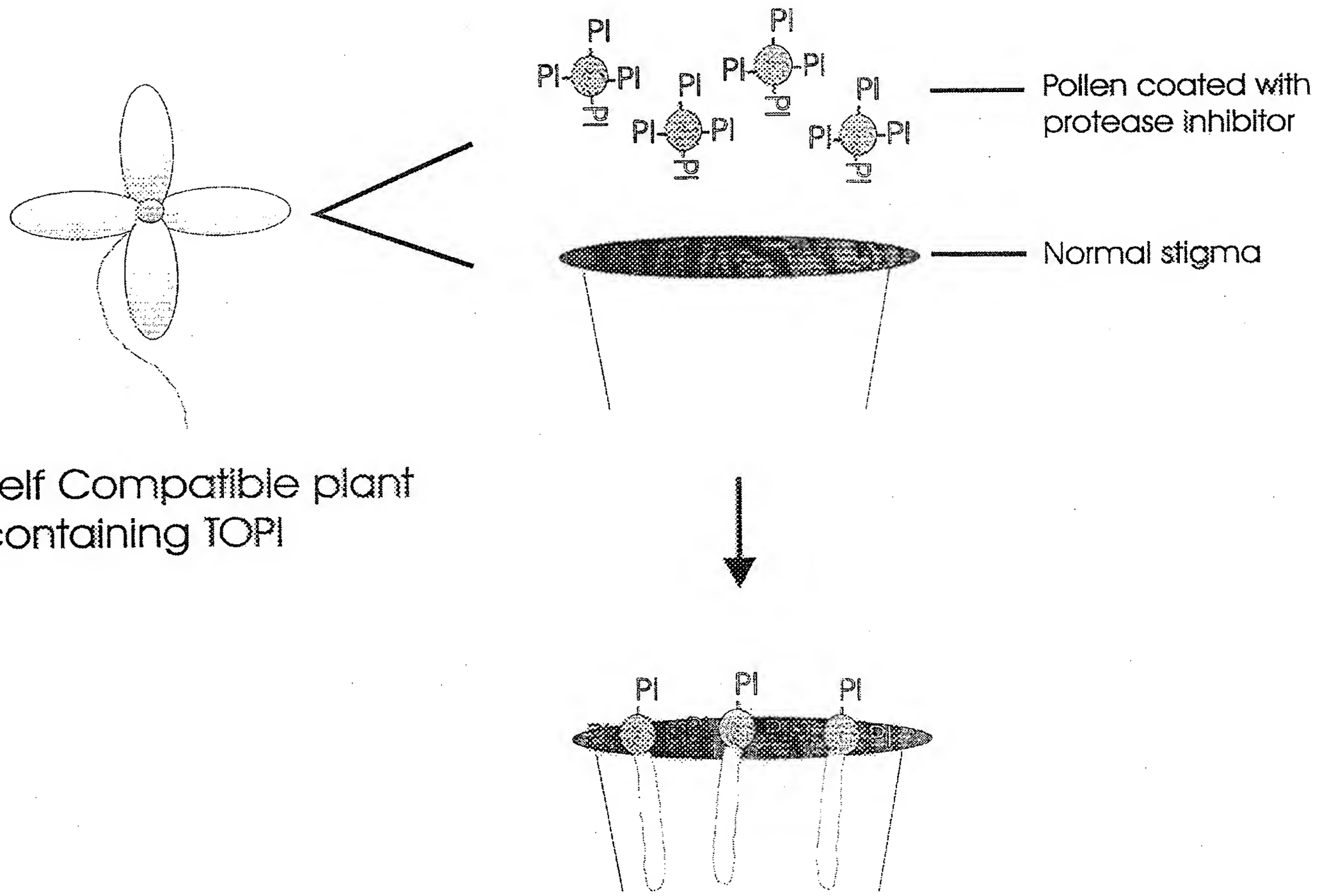

Pollen coated with protease inhibitor
Normal stigma
Self Compatible plant containing TOPI
PI
- Self-fertilization occurs
- Pollen can germinate on stigma expressing protease
- Outcrossing possible
- Incrossing possible

FIG. 15

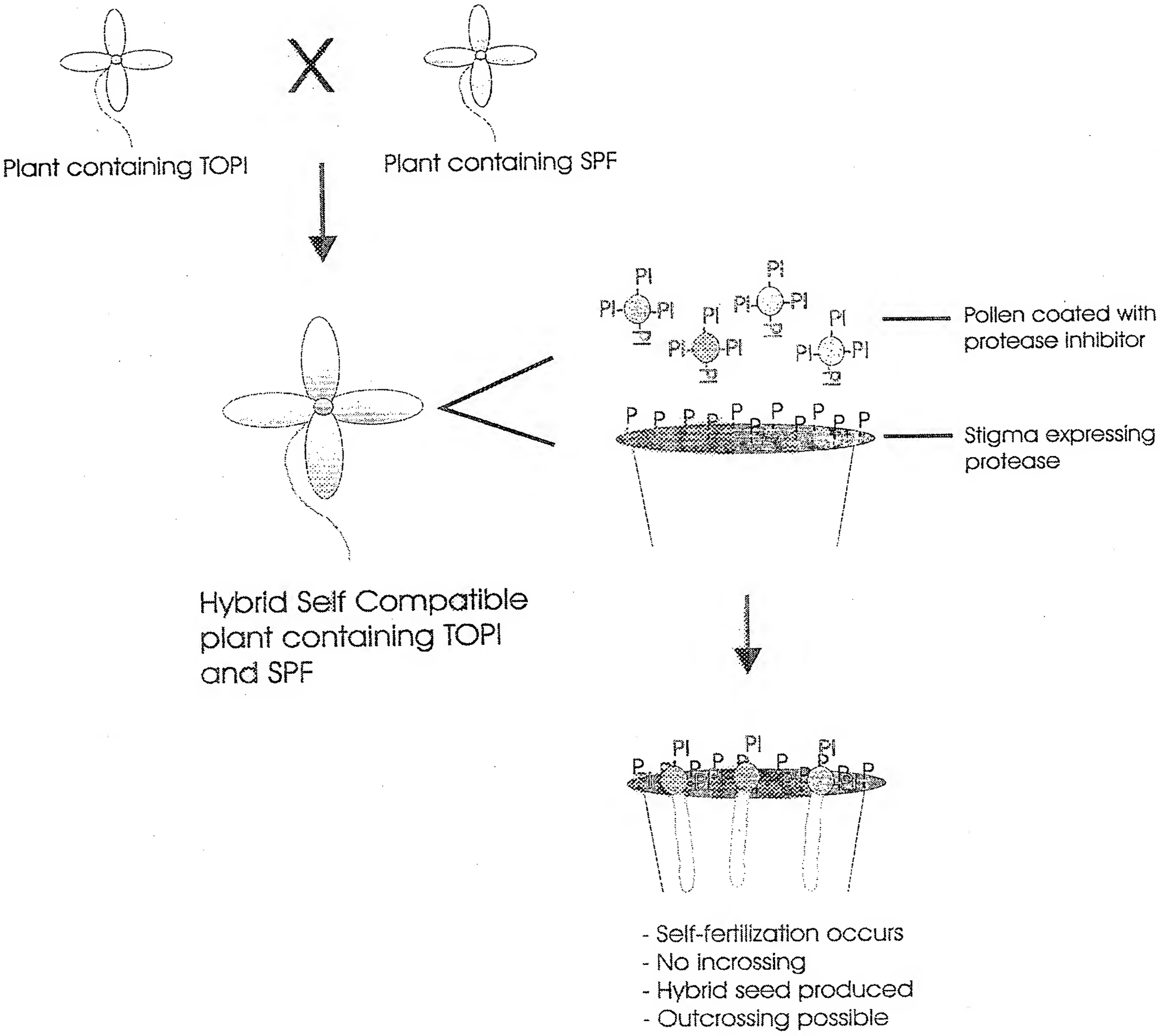

Plant containing TOPI
Plant containing SPF
Pollen coated with protease inhibitor
Stigma expressing protease
Hybrid Self Compatible plant containing TOPI and SPF
- Self-fertilization occurs
- No incrossing
- Hybrid seed produced
- Outcrossing possible

FIG. 16

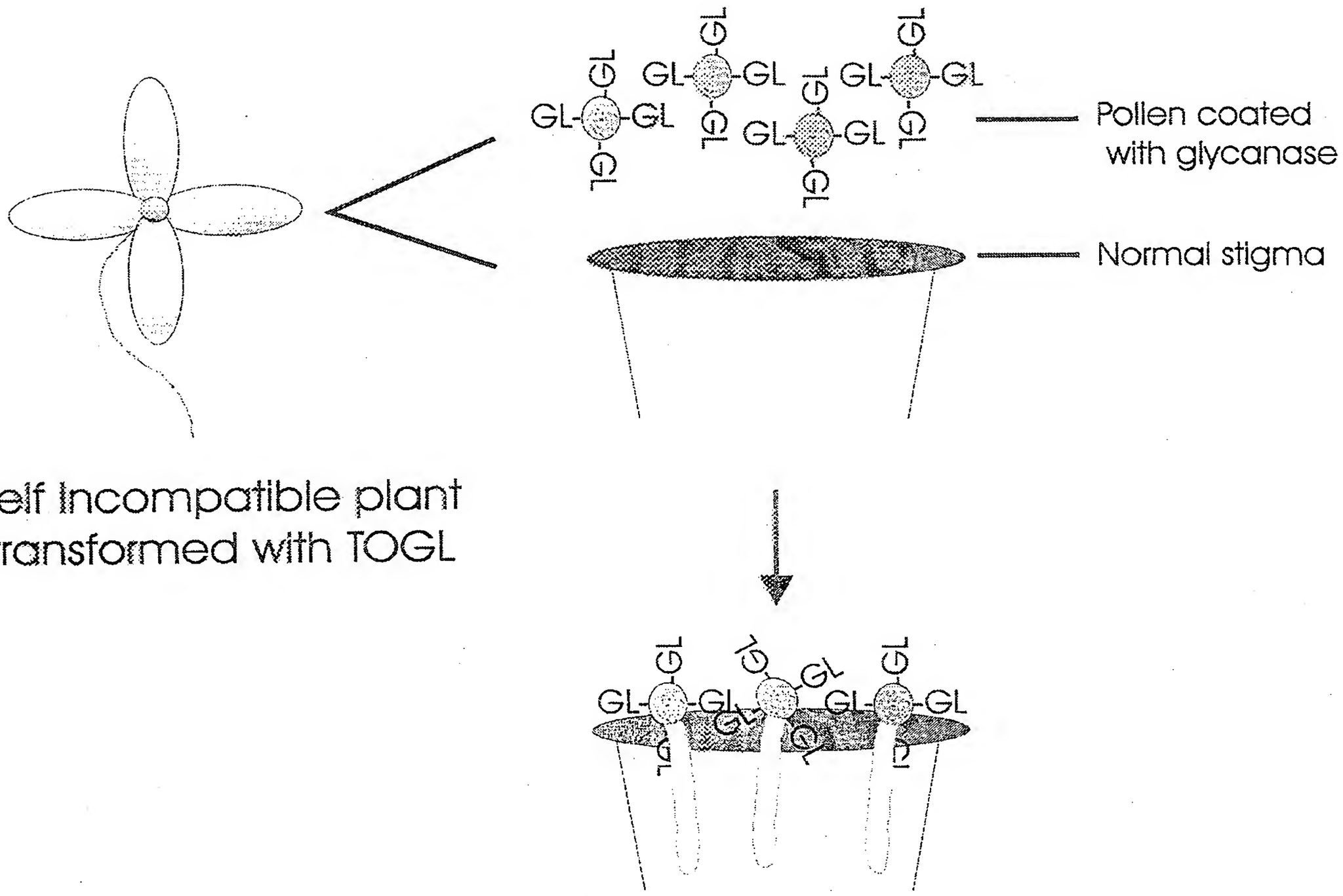

GL
Pollen coated
with glycanase
Normal stigma
Self Incompatible plant
transformed with TOGL
- Self Incompatibility breaks down
- Self-fertilization occurs
- Plant can be maintained

FIG.17

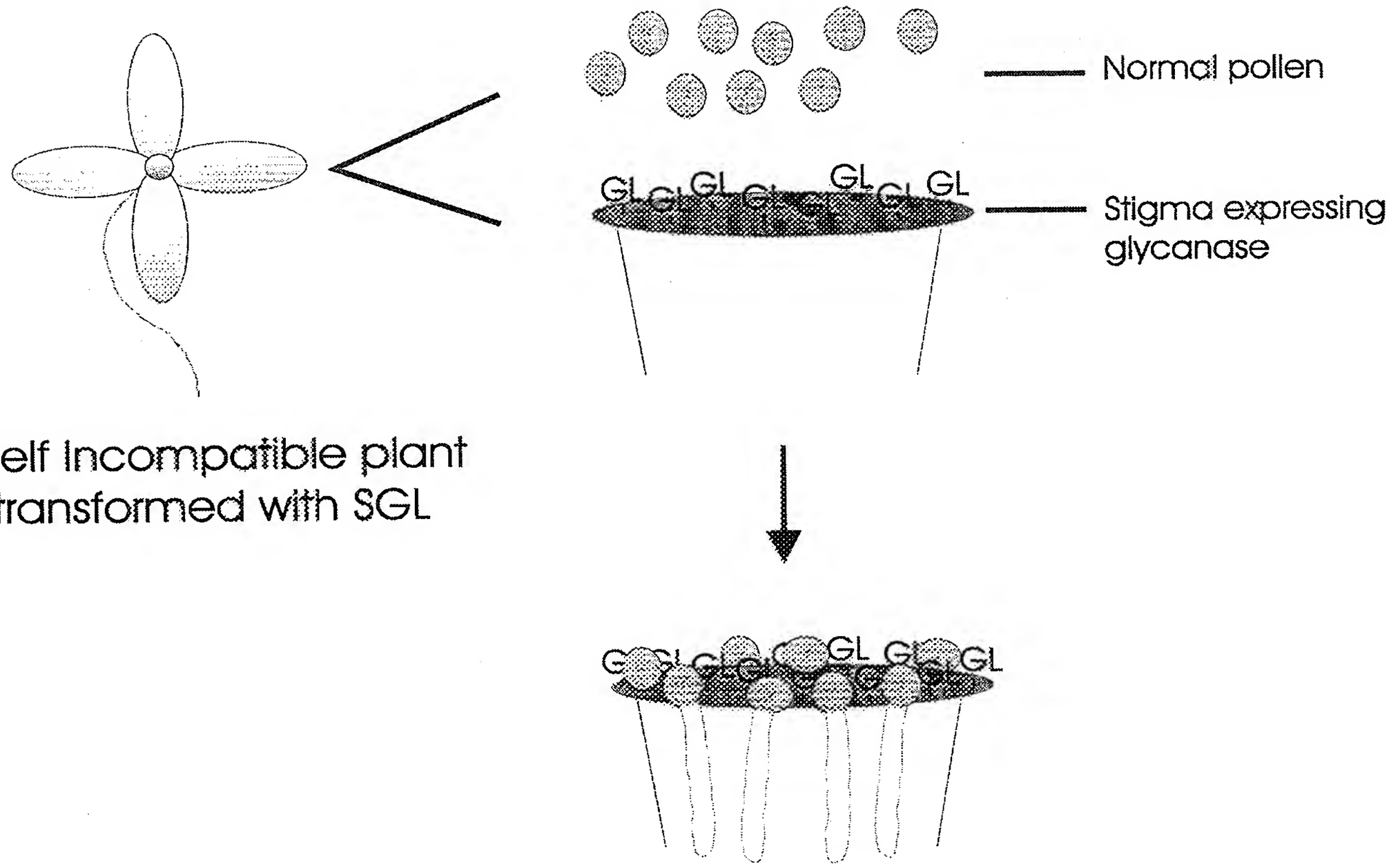

Normal pollen
Stigma expressing glycanase
Self Incompatible plant transformed with SGL
- Self Incompatibility breaks down
- Self-fertilization occurs
- Plant can be maintained

FIG.18

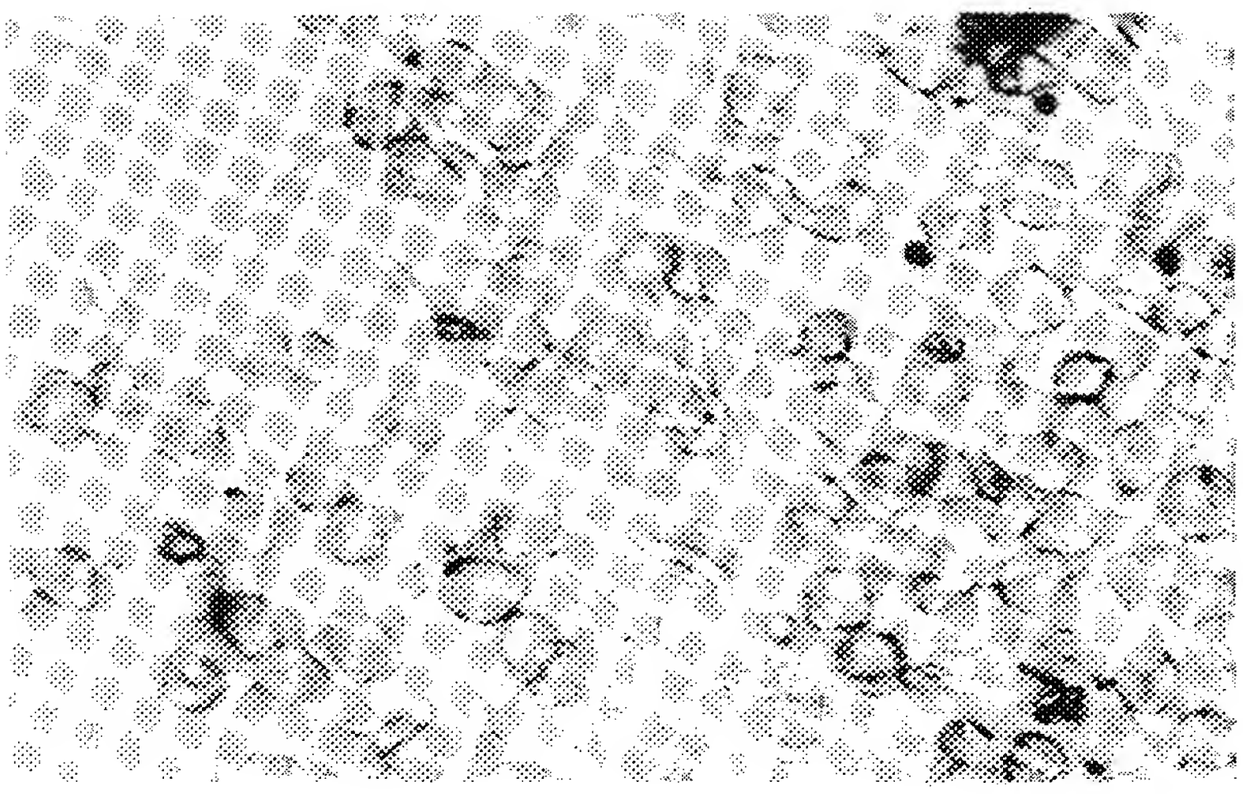


FIG. 19A

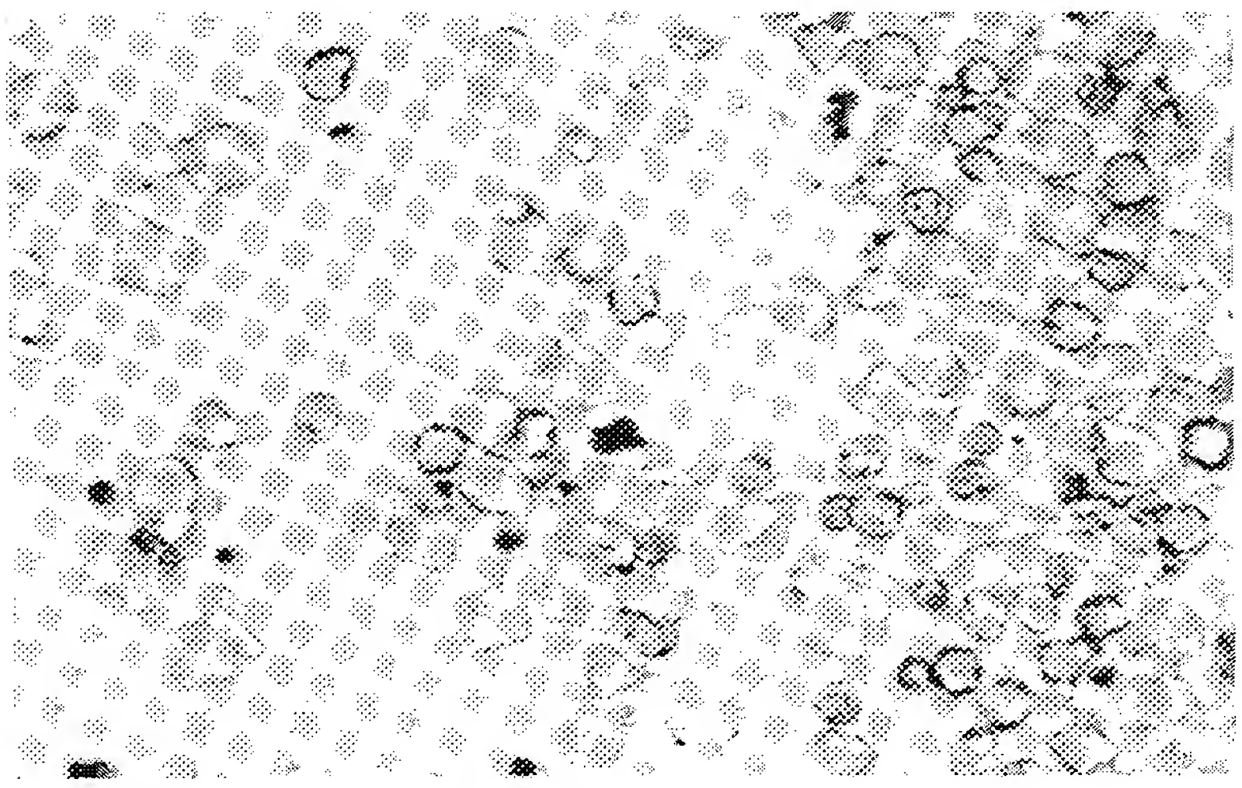


FIG. 19B

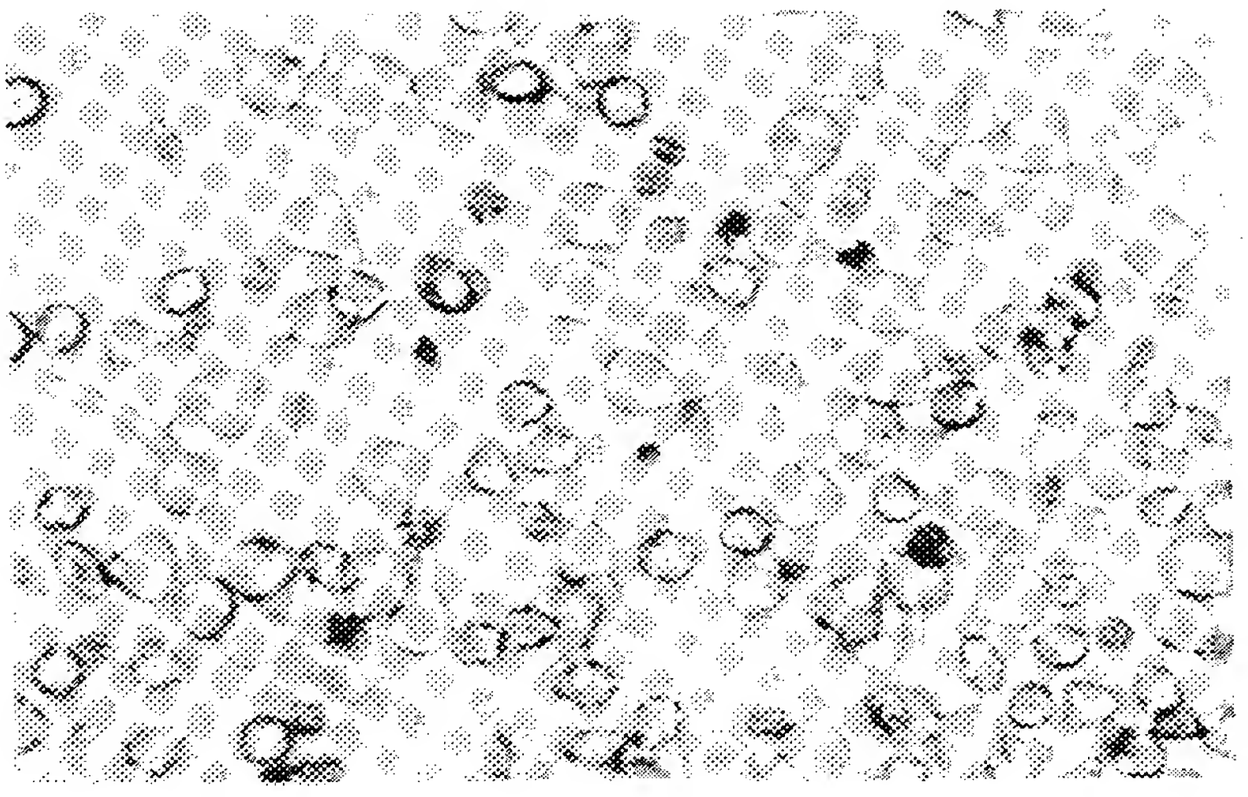


FIG. 19C

FIG. 20 A

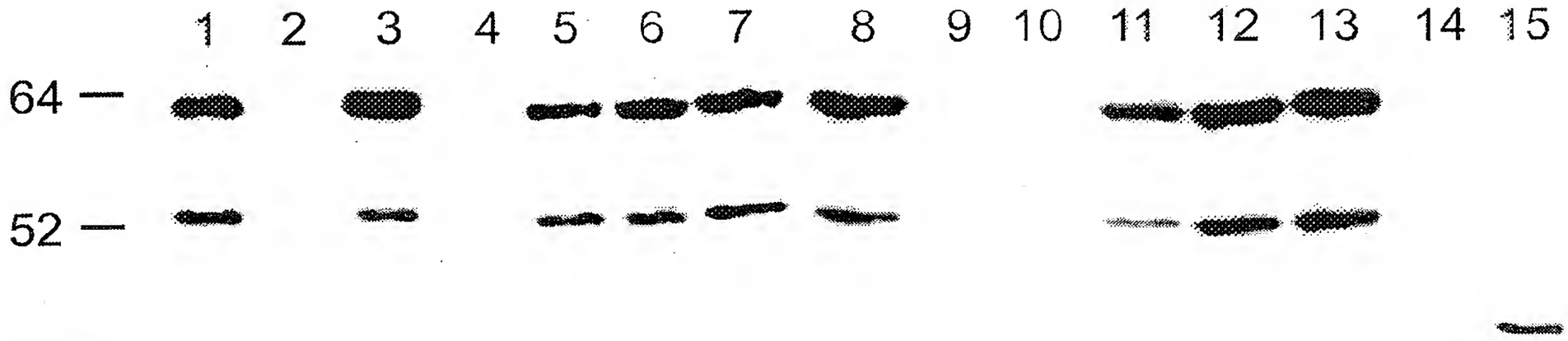


FIG. 20 B

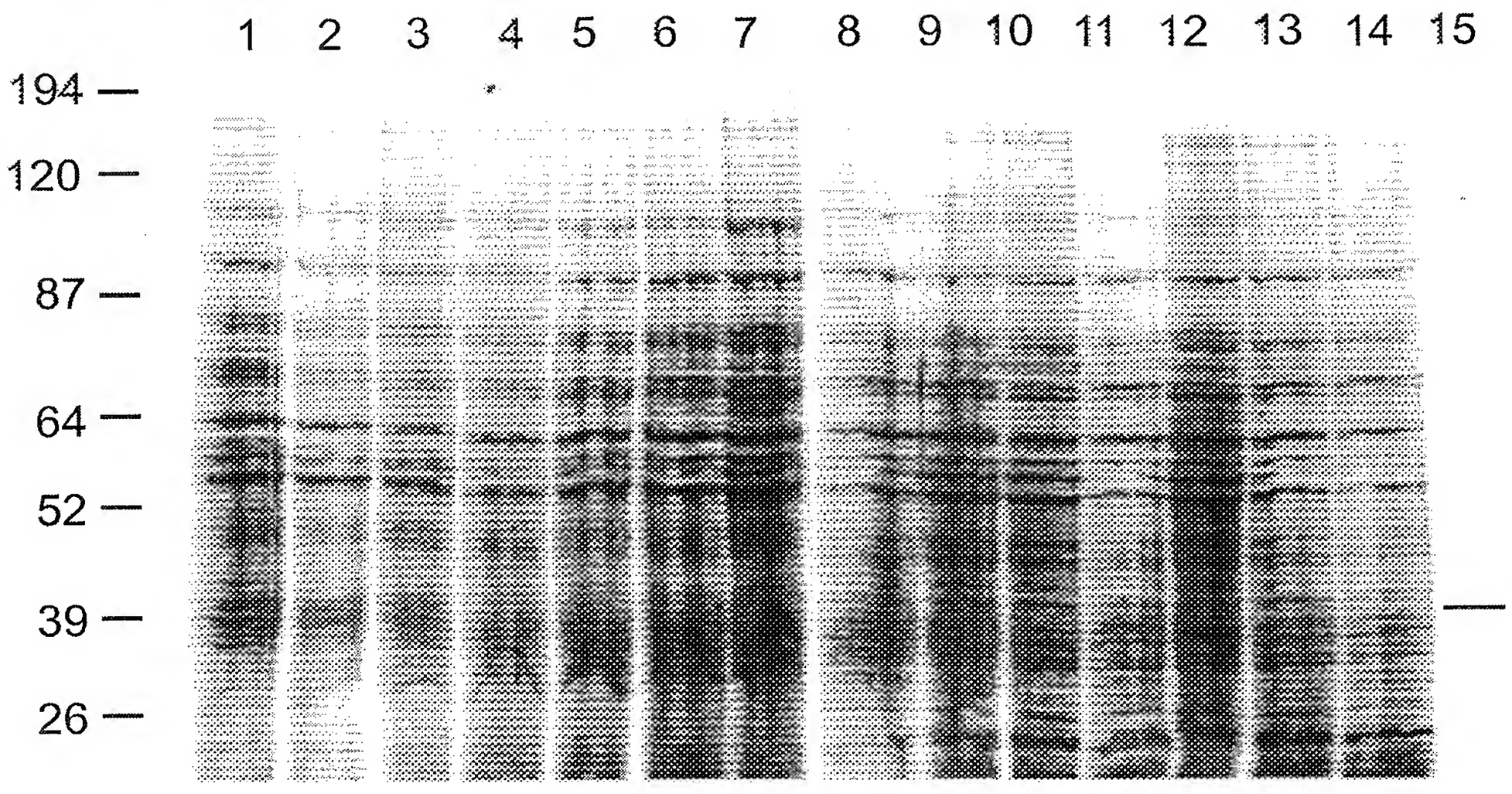


FIG. 20

FIG. 21 A

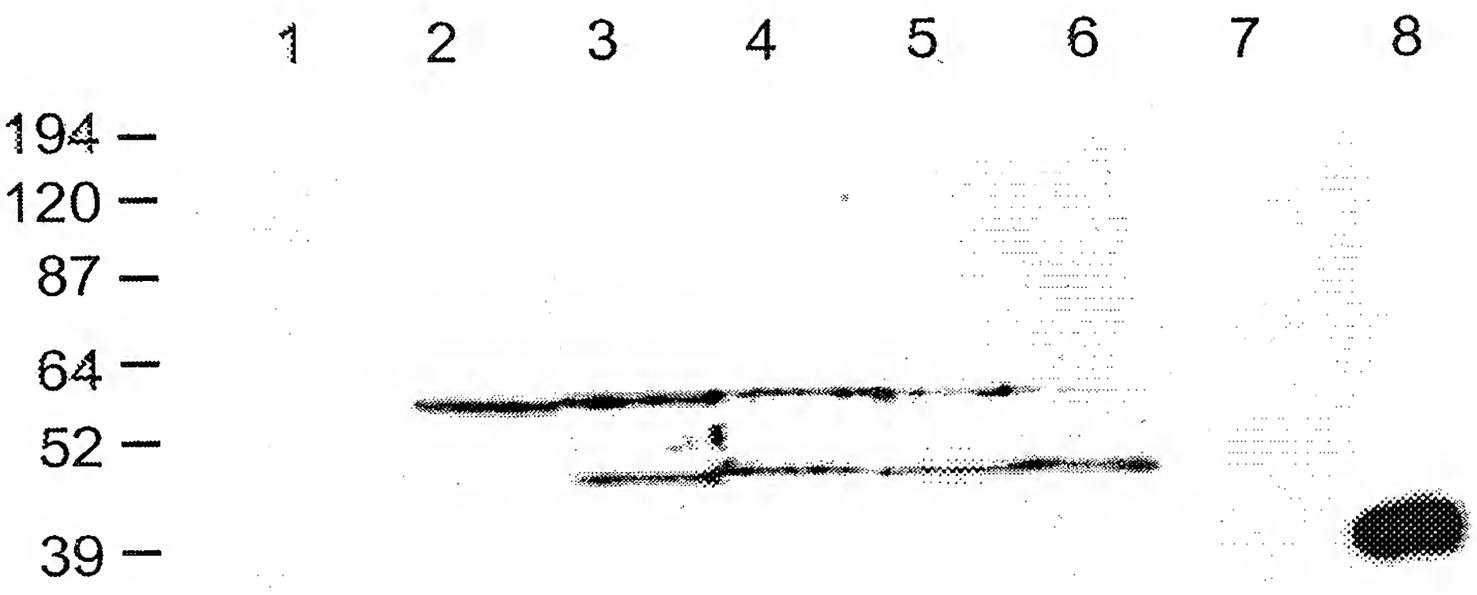


FIG. 21 B

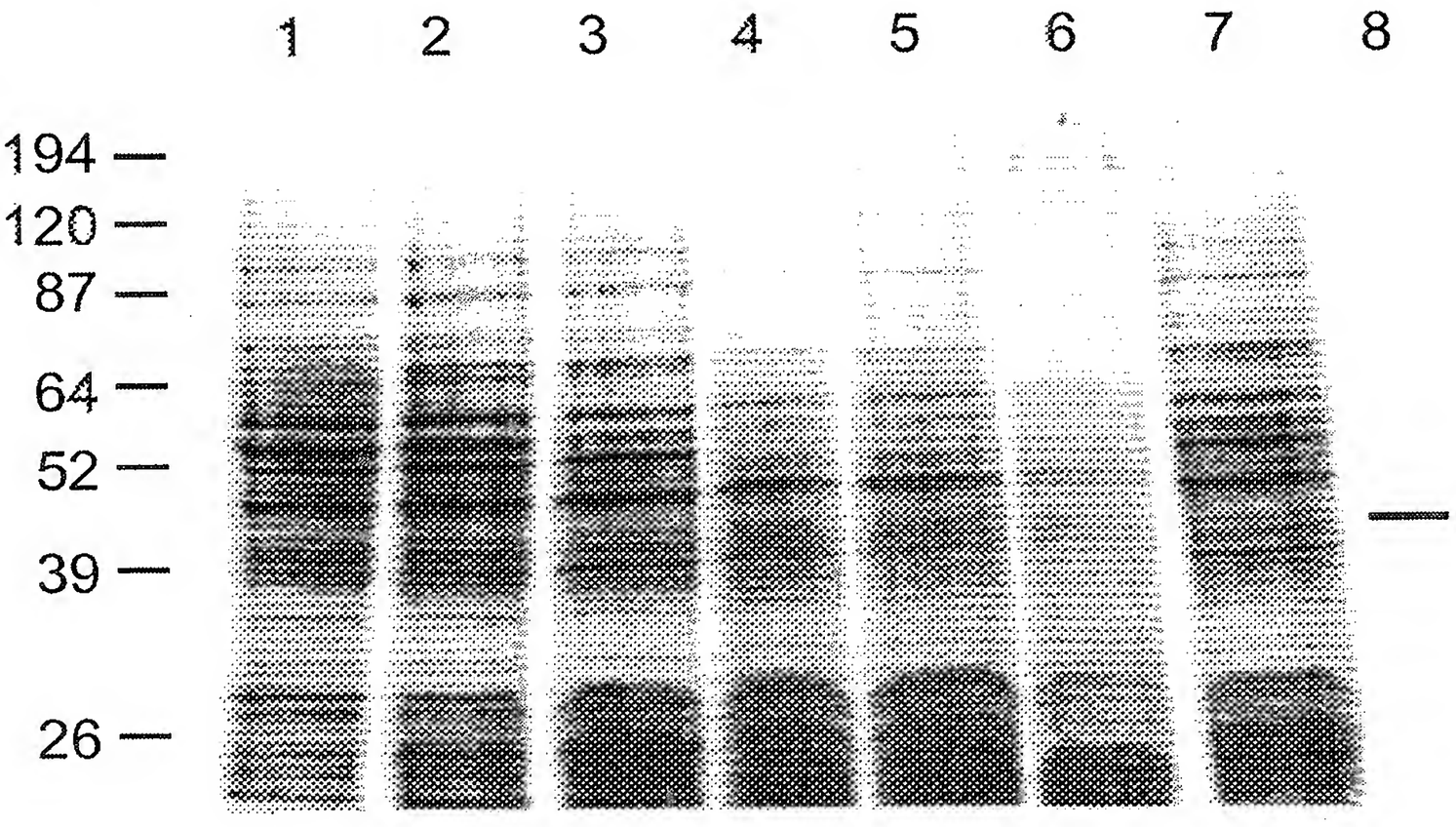
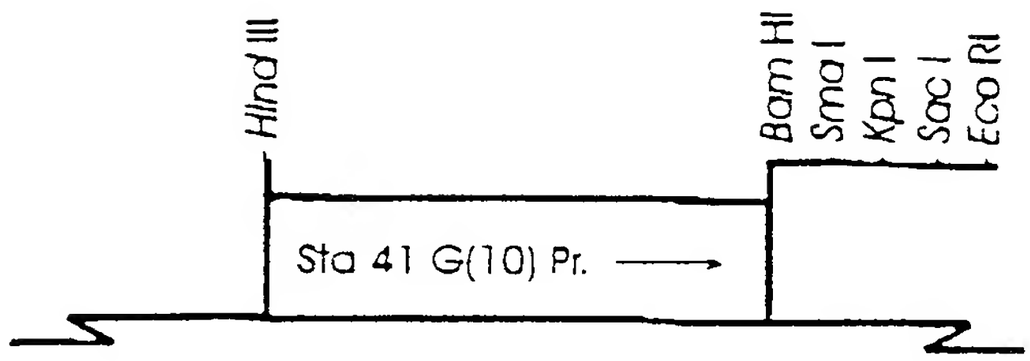
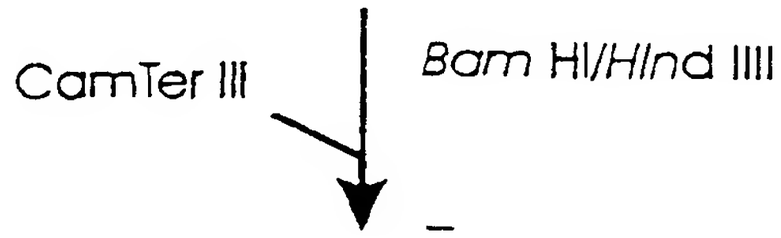
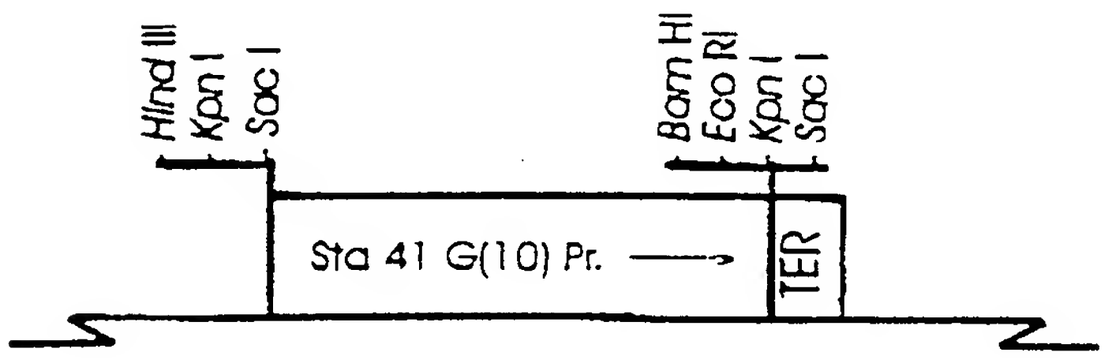


FIG. 21



T28





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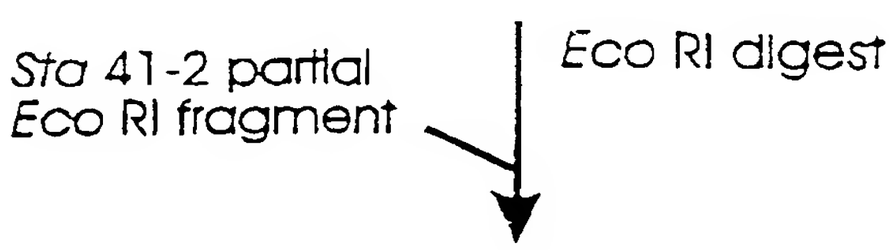

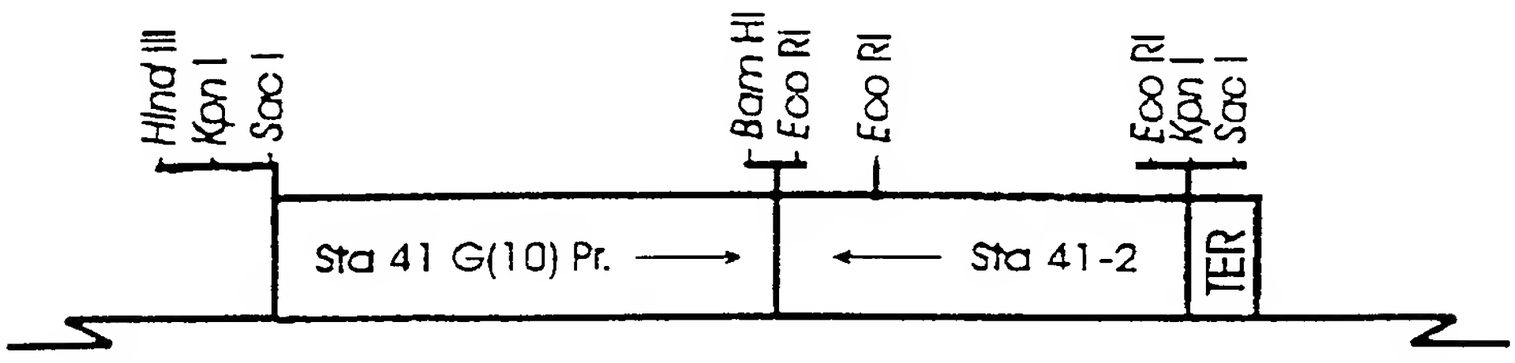

SAS-1

FIG. 22

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Laurian Robert
(B) STREET: 12 De Maison
(C) CITY: Gatineau
(D) STATE: Quebec
(E) COUNTRY: Canada
(F) POSTAL CODE (ZIP): J8V 1Y4

(A) NAME: Stephen Gleddie
(B) STREET: 33 Leonard Ae.
(C) CITY: Ottawa
(D) STATE: Ontarion
(E) COUNTRY: Canada
(F) POSTAL CODE (ZIP): K1S 4T8

(ii) TITLE OF INVENTION: Protein Expression in Floral Cells

(iii) NUMBER OF SEQUENCES: 19

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "KSB-3"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TAGGTACCGA GCTCGGGGGA TCC 23

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "KSB-4"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TAGGATCCCC CGAGCTCGGT ACC 23

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4936 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "translational fusion"

(vii) IMMEDIATE SOURCE:
(B) CLONE: TOG-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GAGCTCCACC CACAGAAGCA GATAAACCAG CTGAAGGAAC AACAGAAAAA CCAAAAGATA 60

ATTCGACTGG AGGAGCAGCC GATAAACCAG AAGATAAACC AGTTGGAGGA GCAGCCGATA 120

AACCAGAAGG TAAACCGGAT GGAGGAGCAA CAAATAAGCC AGAAAGTAAA CCAGCTGGAG 180

GACCATCAAA TAAACCAAAA GATAAACCCG CTGGAGGACC AACGGATAAA CCAGAAAGTA 240

AGCCAGCAGA TAAACCCGCT GGAGGACCAA CAGATAAGCC AGGAAGTAAA CCGGTTGATA 300

AACCCGCTGG AGGACCAACA GATAAGACAG AAAGTAAACT GGTTGGAGAG GCATCAAATA 360

AACCAAAAGA TAAACCCGCT GGTGGATCAA CAGATATGCC AGAAGCTGGA GAGACATCAA 420

ATAAACCAAA AGATAAATCC GTTGGAGGAC CAACAAATAA GCCAGAAAGT AAACCAGCTG 480

GAGAAACATC ACATAAACCA AAAGATAAAC CCGCTGGTGG ACCAACAGAT AAGCCAGAAA 540

GTAAACCGGC TGGAGAAACA TCACATAAAC CAAAAGATAA ACTCGCTGGT GGACCAACAG 600

ATAAGCCAGA AAGTAAACCA GCTGGAGAGG CATCAAATAA ACCAAAAGAT AAACCCGCTG 660

GTGGACCAAC AGATAAACCA GCTGGAGGAT CAGTAGATAA ACCAAAAGAT AAACCTGCCG 720

GAGGACCAAC AGATAAACCA ACAAATAAAC CGACTGGAGG GGCTGCAAAT AAACCGGCTG 780

GAGAGGCAGC AAACAAACCG ACTGGAAAAC CGAAAAATAA ACCGGCTGGA GAGAATAAAC 840

CACCGGGATG GTATAGGTGA ATGGAGTAGT ATGAAATTAA AGTATTGGGT TCCACAAATT 900

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CACGCCGTAT GTTATTGCCG GGAAAAGTGT ACGTATCACC GTTTGTGTGA ACAACGAACT 3540

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GGCGGCTTTT CTGCTGCAAA AACGCTGGAC TGGCATGAAC TTCGGTGAAA AACCGCAGCA 4920

GGGAGGCAAA CAATGA 4936

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3766 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "translational fusion"

(vii) IMMEDIATE SOURCE:
(B) CLONE: TOP-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GAGCTCCACC CACAGAAGCA GATAAACCAG CTGAAGGAAC AACAGAAAAA CCAAAAGATA 60

ATTCGACTGG AGGAGCAGCC GATAAACCAG AAGATAAACC AGTTGGAGGA GCAGCCGATA 120

AACCAGAAGG TAAACCGGAT GGAGGAGCAA CAAATAAGCC AGAAAGTAAA CCAGCTGGAG 180

GACCATCAAA TAAACCAAAA GATAAACCCG CTGGAGGACC AACGGATAAA CCAGAAAGTA 240

AGCCAGCAGA TAAACCCGCT GGAGGACCAA CAGATAAGCC AGGAAGTAAA CCGGTTGATA 300

AACCCGCTGG AGGACCAACA GATAAGACAG AAAGTAAACT GGTTGGAGAG GCATCAAATA 360

AACCAAAAGA TAAACCCGCT GGTGGATCAA CAGATATGCC AGAAGCTGGA GAGACATCAA 420

ATAAACCAAA AGATAAATCC GTTGGAGGAC CAACAAATAA GCCAGAAAGT AAACCAGCTG 480

GAGAAACATC ACATAAACCA AAAGATAAAC CCGCTGGTGG ACCAACAGAT AAGCCAGAAA 540

GTAAACCGGC TGGAGAAACA TCACATAAAC CAAAAGATAA ACTCGCTGGT GGACCAACAG 600

ATAAGCCAGA AAGTAAACCA GCTGGAGAGG CATCAAATAA ACCAAAAGAT AAACCCGCTG 660

GTGGACCAAC AGATAAACCA GCTGGAGGAT CAGTAGATAA ACCAAAAGAT AAACCTGCCG 720

GAGGACCAAC AGATAAACCA ACAAATAAAC CGACTGGAGG GGCTGCAAAT AAACCGGCTG 780

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ATTCCTAATT TATCCTACAC TACATGTTTC ATAATCATTT CTATAAATGT ACGACTTGTT 960

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TATCATTATA ACTGACGTTA GCGTATATCG ACCAATGCGA TAACCAAATC ATCGGTATAT 1080

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CCAATCTTCT TGCATTTTTC ATTGCCATTT ACCTTGAACG CCTCTCTTTC TAGTATGAGA 1200

CATTAACATT GCGCTCTTGT CACAATGAAG CCATGGAAAA CTTCGGCTCT TTAATCACAC 1260

ATGTGACAAT CCAGTTGGTT TAAGGGAAAG TATTTTATAT TTTATATAGC TCGTTCTCAG 1320

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AAGGGAGAAA ATGAAAAGCG TATTTCATAT GAGAGATTGT CAACAAAAAT TGAGTGACTT 1560

TTATGATATT TGTTCAAAGA ATAGTCTAAT AACCTTTCTT ATTTAAATTT TAATTATGTT 1620

ATATATCAAT AATACTAAAA TAATTAGTTA CTCACAGTTC GTGACAAAAA AAAAAGCAAA 1680

TAGATGAAAT GAAATGAAAG AAAGATCTTT CTTCACGCGT TGATATTCAT AAAACAATGG 1740

AATGAAAGAA AACAGTTAAG ATTCTACAAG AAAGAAAAGA AAGTCCCAAA AACATGACAA 1800

ATAGATGAAG AAGCAAATGT GACTTGACGT AACGTAGAAC TCCATATATA CTCCCATCGT 1860

TTTGCATGGA GCATGCATGT GTACCGTGCA CGTCGTAGAC CACACAACTC CTTCATAAAA 1920

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ATCCCTATCT CTGTTGGGCA TACCGGAGAG CATTAAACCA AGTAATATTA TACCGGAGAG 2340

TATTAAACCA AGTAATATTA TACCGGAGGG TATTAAACCA AGTAATATTA AGGACAAAAT 2400

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GGATGACGAT ACGACTACGG ATGAAGACAA ACACGGAAGT GGAGCTAAAC ACGGAAAGGG 2580

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AGGTAAACAC GGAAGCGGAG GTAAACATGA AAGTGGAGGT TCGGCTATGG GTGGAGGTAA 2880

GCACGGAAGT GGAGGCAAAC ACGGAAGTGA AGGTAAACAC GGGGGTGAAG GCTCTTCTAT 2940

GGGTAAAAAT AGTCTATCCA AGAAGAAAAA GGAATTCCAT TATAGAGGTC AAGCTATGGA 3000

TGCAAGTAGT ACAAGTGAAA GTTCAGATGG AAGTTCAGAT GGCAGCAGTT CAGATGGAAG 3060

TTCACATGGG AGTGGTGGTA AACACATAGG TACCGAGCTC GGGGGATCCT TGCCTGATAC 3120

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GTGTTGGAGT TTCAGTGCTA CTGGTTCACT CGAAGGTCAG CACTTCCGTA AAACCGGCAA 3240

ACTAGTGTCC CTTAGCGAAC AAAACTTGGT AGATTGTTCA GGAAGATACG GCAACAACGG 3300

CTGCAACGGC GGTCTCATGG ACAACGCCTT CCGTTACATC AAAGACAACG GCGGTATCGA 3360

CACGGAAAAG TCCTACCCCT ACCTAGCCGA GGACGAGAAA TGCCACTACA AAGCCCAGAA 3420

CAGCGGCGCA ACCGACAAGG GCTTTGTAGA CATCGAAGAA GCCAACGAAG ATGACCTTAA 3480

GGCTGCAGTG GCCACCGTAG GCCCCGTTTC TATTGCCATT GATGCCAGCC ACGAAACCTT 3540

CCAACTGTAC TCGGATGGAG TCTACAGTGA TCCTGAATGT AGCTCACAAG AACTAGACCA 3600

TGGGGTGTTG GTAGTGGGAT ACGGTACCAG CGACGATGGT CAGGACTACT GGTTGGTGAA 3660

AAATTCGTGG GGACCCAGCT GGGGATTGAA CGGATACATC AAGATGGCCA GGAATCAAGA 3720

TAACATGTGC GGAGTTGCAT CTCAGGCTAG TTATCCTTTG GTTTAG 3766

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3496 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "tranlational fusion"

(vii) IMMEDIATE SOURCE:
(B) CLONE: TOPI-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GAGCTCCACC CACAGAAGCA GATAAACCAG CTGAAGGAAC AACAGAAAAA CCAAAAGATA 60

ATTCGACTGG AGGAGCAGCC GATAAACCAG AAGATAAACC AGTTGGAGGA GCAGCCGATA 120

AACCAGAAGG TAAACCGGAT GGAGGAGCAA CAAATAAGCC AGAAAGTAAA CCAGCTGGAG 180

GACCATCAAA TAAACCAAAA GATAAACCCG CTGGAGGACC AACGGATAAA CCAGAAAGTA 240

AGCCAGCAGA TAAACCCGCT GGAGGACCAA CAGATAAGCC AGGAAGTAAA CCGGTTGATA 300

AACCCGCTGG AGGACCAACA GATAAGACAG AAAGTAAACT GGTTGGAGAG GCATCAAATA 360

AACCAAAAGA TAAACCCGCT GGTGGATCAA CAGATATGCC AGAAGCTGGA GAGACATCAA 420

ATAAACCAAA AGATAAATCC GTTGGAGGAC CAACAAATAA GCCAGAAAGT AAACCAGCTG 480

GAGAAACATC ACATAAACCA AAAGATAAAC CCGCTGGTGG ACCAACAGAT AAGCCAGAAA 540

GTAAACCGGC TGGAGAAACA TCACATAAAC CAAAAGATAA ACTCGCTGGT GGACCAACAG 600

ATAAGCCAGA AAGTAAACCA GCTGGAGAGG CATCAAATAA ACCAAAAGAT AAACCCGCTG 660

GTGGACCAAC AGATAAACCA GCTGGAGGAT CAGTAGATAA ACCAAAAGAT AAACCTGCCG 720

GAGGACCAAC AGATAAACCA ACAAATAAAC CGACTGGAGG GGCTGCAAAT AAACCGGCTG 780

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CACCGGGATG GTATAGGTGA ATGGAGTAGT ATGAAATTAA AGTATTGGGT TCCACAAATT 900

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TAATGTTCTG GGCTAGCCCA TTTGAATTTA AACCTTTCCT TTCAATTTCT GCATTAATAT 1440

AATTCAGTTG TTCAAAAAAA AAATAGCGCT TATTGAAATA ATAGAGAGAA AGATAATGAG 1500

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TTTGCATGGA GCATGCATGT GTACCGTGCA CGTCGTAGAC CACACAACTC CTTCATAAAA 1920

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ACAATCAAAC GATGAATATC ATTTGATGCC GATCAAATTA CTGAAGGTTT CATCTCAAGT 3300

TGTCGCTGGT GTGAAATACA AGATGGATGT GCAGGTTGCT CGATCGCAAT GTAAAAAAAG 3360

TTCGAATGAA AAAGTTGATC TAACAAAGTG CAAAAAATTA GAAGGACATC CTGAAAAGGT 3420

TATGACTTTG GAAGTTTGGG AGAAACCATG GGAGAATTTT ATGCGCGTCG AAATTCTGGG 3480

AACAAAAGAA GTATGA 3496

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "BKX-1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TCGAGGGGAT CCGGTACCTC TAGA 24

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "BKX-2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

TCGATCTAGA GGTACCGGAT CCCC 24

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3459 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "translational fusion"

(vii) IMMEDIATE SOURCE:
(B) CLONE: SPF-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GGGGATCTAT CTTAGATATT GTTTGAAAAG AGAGTACCAA CTCTCCCATA CTCAAAGTCA 60

CAGAAGTCAA ATGTTGTTTG AAATAATTAT AGCATTTGTT TGTTTTAGTT AAAAGTTTGA 120

AAAACTACGA ACGATTTATG GATCATCATT CATCACGCAT ATTGTTAATG TGAGAACCAG 180

ACCAATAGTT TCTTAAGTAT TGAATATACA AAAAACATTT GATAAACAAA TCTTTCGCTT 240

TGGAAAAATA TAGGGTGGGT AATATTTTGT ATAATCTAAG CGGCTTGTTC ATTTGTAATC 300

ATGGATTTTA ATGTTTTTGA TGACTTTGTT TGTGGATGTG CAACTGCATA TTTTTAGTTT 360

TTTTTGTTTG TTAATTGTGT TGTGATCCTT AATCCTAATT ATGAAACACA CAAGTATGAC 420

TGATAAGAGT TAGTAAGCAT TCGGTGGTCA AGATATTTAG TGGCTAGCGA CCTACCCCTA 480

ATTTATTTAT TTATTTTAAC TAATCTTTAT TTGATCGTTC ATTATGTCAA CAAACTTTCT 540

TCTTCTCTAA AGTACGTGAA ATTACTACTA CATTTTAATA CTGCAATTGA AGTAAGTAGT 600

ACGGTTTTCA TCTCTTAAAT GCATGGAAAC AAACACTAAT ACGTAGCAAA ATTGAGAGAA 660

CATATACTAT GCTTCGCACC GGATTTTATT AGAGCATTTG ACACACTAAA GTCCCACATG 720

GTTACCAGCA GGGCCTGCCC TGAGATTTAG AGGGAGGTGG TTTTTAAAAA AAATTCCGTT 780

AAAAATTTTT TTTGGTAAAT TTGGAGGTCT AAAAAAAAAT TTTAAAAGTT TTTTCCTATG 840

TAATTTTTTC CAAAATTTTT AGAGGTCTAA GCCTAATATT TCGTTAGGCT TTAAGCAGGA 900

CCGGCCCTGG TTACCATATG TATAATACAT ACACAAAACA CAATTGTGTT GTCGTTTTTA 960

GTACTTTGTG TCCGTTTCAT TGTATATGAC GTACGTAACC ATTCAAAACC TAATTAAATA 1020

TGGTGATCCC CTAATTGATC ACATTCTAAG CTCTGGTAAA CTTCTCATGG CATACTCTTT 1080

TGATTTCGTA AACCCTTTCT CAAAAAGCTA TTTTCGTATT AATTTGGTAA GAATTATTTT 1140

CCTGGTCCAT GTAGGTTTTG TATGTTTTTT TTTTTGATAA CTCTGGTATC TGGGCAGCCA 1200

CATTCCCAAC TATCCATTCG AAAGGGGTCC AGCGCCCCGG GATGTTAAAT CCTGTTGTGG 1260

CCAAGGCTCG AACCCGGGTA GCGGCAGTAC AACCATACCT CCTTTACCAC CAAGTTACGA 1320

GTGTTTGGTT AAGTTTTGTA TGTTGATGCA GCGTGTGAGT ATCTTAGACT CTTAGTGTCT 1380

CCAGCCCCAT ATTCTTTTAA AACATGGAGG ACTGATCACT ATCCATAACT CTTTTAACAA 1440

AACTACACAC AAGAAATACA CATTTTTCAT CTCACACACA GTTTTAGGTA TACTAATATT 1500

TAATGTAATT AAAGGTTTTC TTAGTTATCA TATTTTGGGA TATAATAAAA ACTGTATAGG 1560

TTGAACTTTT TATAGTGACT GGGCTCACTC CGAAGGGTCA CCTTGCCAGA AATCTCCGTA 1620

GGGATTTTTT AGCTAACCCA GTACCACCCC GCTGTCCCTG AGTATCGAAC TGGCGACCTC 1680

GGGTAATCGT GTGTGAGAAA CGTTCAGTAC TGCCGCTAGG CACCTGACGT TCTCAAAAAA 1740

ACTGTATAGG TTGAACTTTT TTTAATAACT AGGCTCACCC CGGAGATCAC CTCGCCAGAA 1800

ATCCCCGTAG ATATAATAAC CATATAGCCT CCAAAAGTGG AGCAATTTCT TTGAAACGCA 1860

TCTCATCCAT ATAGAGACCA ACATTAACCA TTATCACCAA TTCACTCTTT ATTTCCACCT 1920

AACCATTTAA AAGTCTATAT ATATATATAT ATATATATAT ATATATGTTA AAGGAGCTAA 1980

ATTAATCAAA AATGATAAAC ATCTAATATA TCCTATTCTC CTATATATAG ACACTCCCAC 2040

TAACTCTCAC AGACCCACAA CACTCACACC ATCATGGCTT TCTCCACTAG ATCAAGACTC 2100

TTCCTCTTGT TCTTAACACT TCTTCCTTTC TCCACTCAAA TCAATGCAAG AGAGAGCTAT 2160

TCCTTTGGAA AGTTCCAGAG AGAATACCCC AAAGATCAAA ACCCTAATAA TCTCCAAACC 2220

AACGAGACGA GCGAGCAAGA CGACCAGAAC CCTCCCTTTA TGCCCCAGTC CGGAAACGGG 2280

TACGGCTTGT ATGGTCAAGA AACAACCTAC AACAACAATG AAGAGCAGTT GAATAACAAA 2340

AAATACGACG AGAACGTTAA CTACGACGAC TCTTTCTCAA CCCCAAGCCT AAGCCAAACC 2400

CAAGAATCTT ACAAGACATA TGGAGACAGC TATCCTAAGA CGACCGAGAG TTACAACGAG 2460

AACAACAAGG ACACAAGCTA CTACGAAAAC TCCAATGGCT ACGGGCCAGA GAATAGAGAG 2520

GAGGATGCGT ACAAGGGTTA TGGCAACAAC GTGGAGAGAC AAAGGATGAG CGATAAGAGC 2580

TACTACGAAA ACTCCAATGG TTACGAGCCG GAGAAGAGAG AGAAGGAGGC GTACAAGGGC 2640

TACAGGAACA ATGTGGAGAG ACAAGGGATG AGCGATACGA GGTTCATGGC CAATGGTAAG 2700

TACTACTATG ACCTTGATGA CGACAGAAAC CACGGCCGTT TCTACCAGAA GCATTACTAC 2760

AGCTACAACC CCACCAGTTA CAATGAGGAC TCGAGGGGAT CCTTGCCTGA TACTGTTGAC 2820

TGGAGGGACA AAGGAGCTGT CACTGAAGTC AAAGACCAAG GTCACTGCGG GTCGTGTTGG 2880

AGTTTCAGTG CTACTGGTTC ACTCGAAGGT CAGCACTTCC GTAAAACCGG CAAACTAGTG 2940

TCCCTTAGCG AACAAAACTT GGTAGATTGT TCAGGAAGAT ACGGCAACAA CGGCTGCAAC 3000

GGCGGTCTCA TGGACAACGC CTTCCGTTAC ATCAAAGACA ACGGCGGTAT CGACACGGAA 3060

AAGTCCTACC CCTACCTAGC CGAGGACGAG AAATGCCACT ACAAAGCCCA GAACAGCGGC 3120

GCAACCGACA AGGGCTTTGT AGACATCGAA GAAGCCAACG AAGATGACCT TAAGGCTGCA 3180

GTGGCCACCG TAGGCCCCGT TTCTATTGCC ATTGATGCCA GCCACGAAAC CTTCCAACTG 3240

TACTCGGATG GAGTCTACAG TGATCCTGAA TGTAGCTCAC AAGAACTAGA CCATGGGGTG 3300

TTGGTAGTGG GATACGGTAC CAGCGACGAT GGTCAGGACT ACTGGTTGGT GAAAAATTCG 3360

TGGGGACCCA GCTGGGGATT GAACGGATAC ATCAAGATGG CCAGGAATCA AGATAACATG 3420

TGCGGAGTTG CATCTCAGGC TAGTTATCCT TTGGTTTAG 3459

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3210 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "translational fusion"

(vii) IMMEDIATE SOURCE:
(B) CLONE: SPIF-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GGGGATCTAT CTTAGATATT GTTTGAAAAG AGAGTACCAA CTCTCCCATA CTCAAAGTCA 60

CAGAAGTCAA ATGTTGTTTG AAATAATTAT AGCATTTGTT TGTTTTAGTT AAAAGTTTGA 120

AAAACTACGA ACGATTTATG GATCATCATT CATCACGCAT ATTGTTAATG TGAGAACCAG 180

ACCAATAGTT TCTTAAGTAT TGAATATACA AAAAACATTT GATAAACAAA TCTTTCGCTT 240

TGGAAAAATA TAGGGTGGGT AATATTTTGT ATAATCTAAG CGGCTTGTTC ATTTGTAATC 300

ATGGATTTTA ATGTTTTTGA TGACTTTGTT TGTGGATGTG CAACTGCATA TTTTTAGTTT 360

TTTTTGTTTG TTAATTGTGT TGTGATCCTT AATCCTAATT ATGAAACACA CAAGTATGAC 420

TGATAAGAGT TAGTAAGCAT TCGGTGGTCA AGATATTTAG TGGCTAGCGA CCTACCCCTA 480

ATTTATTTAT TTATTTTAAC TAATCTTTAT TTGATCGTTC ATTATGTCAA CAAACTTTCT 540

TCTTCTCTAA AGTACGTGAA ATTACTACTA CATTTTAATA CTGCAATTGA AGTAAGTAGT 600

ACGGTTTTCA TCTCTTAAAT GCATGGAAAC AAACACTAAT ACGTAGCAAA ATTGAGAGAA 660

CATATACTAT GCTTCGCACC GGATTTTATT AGAGCATTTG ACACACTAAA GTCCCACATG 720

GTTACCAGCA GGGCCTGCCC TGAGATTTAG AGGGAGGTGG TTTTTAAAAA AAATTCCGTT 780

AAAAATTTTT TTTGGTAAAT TTGGAGGTCT AAAAAAAAAT TTTAAAAGTT TTTTCCTATG 840

TAATTTTTTC CAAAATTTTT AGAGGTCTAA GCCTAATATT TCGTTAGGCT TTAAGCAGGA 900

CCGGCCCTGG TTACCATATG TATAATACAT ACACAAAACA CAATTGTGTT GTCGTTTTTA 960

GTACTTTGTG TCCGTTTCAT TGTATATGAC GTACGTAACC ATTCAAAACC TAATTAAATA 1020

TGGTGATCCC CTAATTGATC ACATTCTAAG CTCTGGTAAA CTTCTCATGG CATACTCTTT 1080

TGATTTCGTA AACCCTTTCT CAAAAAGCTA TTTTCGTATT AATTTGGTAA GAATTATTTT 1140

CCTGGTCCAT GTAGGTTTTG TATGTTTTTT TTTTTGATAA CTCTGGTATC TGGGCAGCCA 1200

CATTCCCAAC TATCCATTCG AAAGGGGTCC AGCGCCCCGG GATGTTAAAT CCTGTTGTGG 1260

CCAAGGCTCG AACCCGGGTA GCGGCAGTAC AACCATACCT CCTTTACCAC CAAGTTACGA 1320

GTGTTTGGTT AAGTTTTGTA TGTTGATGCA GCGTGTGAGT ATCTTAGACT CTTAGTGTCT 1380

CCAGCCCCAT ATTCTTTTAA AACATGGAGG ACTGATCACT ATCCATAACT CTTTTAACAA 1440

AACTACACAC AAGAAATACA CATTTTTCAT CTCACACACA GTTTTAGGTA TACTAATATT 1500

TAATGTAATT AAAGGTTTTC TTAGTTATCA TATTTTGGGA TATAATAAAA ACTGTATAGG 1560

TTGAACTTTT TATAGTGACT GGGCTCACTC CGAAGGGTCA CCTTGCCAGA AATCTCCGTA 1620

GGGATTTTTT AGCTAACCCA GTACCACCCC GCTGTCCCTG AGTATCGAAC TGGCGACCTC 1680

GGGTAATCGT GTGTGAGAAA CGTTCAGTAC TGCCGCTAGG CACCTGACGT TCTCAAAAAA 1740

ACTGTATAGG TTGAACTTTT TTTAATAACT AGGCTCACCC CGGAGATCAC CTCGCCAGAA 1800

ATCCCCGTAG ATATAATAAC CATATAGCCT CCAAAAGTGG AGCAATTTCT TTGAAACGCA 1860

TCTCATCCAT ATAGAGACCA ACATTAACCA TTATCACCAA TTCACTCTTT ATTTCCACCT 1920

AACCATTTAA AAGTCTATAT ATATATATAT ATATATATAT ATATATGTTA AAGGAGCTAA 1980

ATTAATCAAA AATGATAAAC ATCTAATATA TCCTATTCTC CTATATATAG ACACTCCCAC 2040

TAACTCTCAC AGACCCACAA CACTCACACC ATCATGGCTT TCTCCACTAG ATCAAGACTC 2100

TTCCTCTTGT TCTTAACACT TCTTCCTTTC TCCACTCAAA TCAATGCAAG AGAGAGCTAT 2160

TCCTTTGGAA AGTTCCAGAG AGAATACCCC AAAGATCAAA ACCCTAATAA TCTCCAAACC 2220

AACGAGACGA GCGAGCAAGA CGACCAGAAC CCTCCCTTTA TGCCCCAGTC CGGAAACGGG 2280

TACGGCTTGT ATGGTCAAGA AACAACCTAC AACAACAATG AAGAGCAGTT GAATAACAAA 2340

AAATACGACG AGAACGTTAA CTACGACGAC TCTTTCTCAA CCCCAAGCCT AAGCCAAACC 2400

CAAGAATCTT ACAAGACATA TGGAGACAGC TATCCTAAGA CGACCGAGAG TTACAACGAG 2460

AACAACAAGG ACACAAGCTA CTACGAAAAC TCCAATGGCT ACGGGCCAGA GAATAGAGAG 2520

GAGGATGCGT ACAAGGGTTA TGGCAACAAC GTGGAGAGAC AAAGGATGAG CGATAAGAGC 2580

TACTACGAAA ACTCCAATGG TTACGAGCCG GAGAAGAGAG AGAAGGAGGC GTACAAGGGC 2640

TACAGGAACA ATGTGGAGAG ACAAGGGATG AGCGATACGA GGTTCATGGC CAATGGTAAG 2700

TACTACTATG ACCTTGATGA CGACAGAAAC CACGGCCGTT TCTACCAGAA GCATTACTAC 2760

AGCTACAACC CCACCAGTTA CAATGAGGAC TCGAGGGGAT CCGGTACCCA ATTCCGCAAA 2820

AATCCGTCAA AAATGGAGTC CAAAACAGGT GAAAATCAAG ATCGTCCCGT TTTATTGGGA 2880

GGTTGGGAAG ATCGCGATCC AAAGGATGAA GAAATCCTGG AACTATTGCC AAGCATATTG 2940

ATGAAAGTAA ATGAACAATC AAACGATGAA TATCATTTGA TGCCGATCAA ATTACTGAAG 3000

GTTTCATCTC AAGTTGTCGC TGGTGTGAAA TACAAGATGG ATGTGCAGGT TGCTCGATCG 3060

CAATGTAAAA AAAGTTCGAA TGAAAAAGTT GATCTAACAA AGTGCAAAAA ATTAGAAGGA 3120

CATCCTGAAA AGGTTATGAC TTTGGAAGTT TGGGAGAAAC CATGGGAGAA TTTTATGCGC 3180

GTCGAAATTC TGGGAACAAA AGAAGTATGA 3210

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "SLG26 (7)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

ATAGAGCTCC GATGAAAGGC ATAAGAA 27

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "SLG26 (8)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

TATGGTACCT TCTTCAGAAG ACAAAGTG 28

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1334 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "tranlational fusion"

(vii) IMMEDIATE SOURCE:
(B) CLONE: SPOV-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GTCAACATGG TGGAGCACGA CACTCTCGTC TACTCCAAGA ATATCAAAGA TACAGTCTCA 60

GAAGACCAAA GGGCTATTGA GACTTTTCAA CAAAGGGTAA TATCGGGAAA CCTCCTCGGA 120

TTCCATTGCC CAGCTATCTG TCACTTCATC AAAAGGACAG TAGAAAAGGA AGGTGGCACC 180

TACAAATGCC ATCATTGCGA TAAAGGAAAG GCTATCGTTC AAGATGCCTC TGCCGACAGT 240

GGTCCCAAAG ATGGACCCCC ACCCACGAGG AGCATCGTGG AAAAAGAAGA CGTTCCAACC 300

ACGTCTTCAA AGCAAGTGGA TTGATGTGAT AACATGGTGG AGCACGACAC TCTCGTCTAC 360

TCCAAGAATA TCAAAGATAC AGTCTCAGAA GACCAAAGGG CTATTGAGAC TTTTCAACAA 420

AGGGTAATAT CGGGAAACCT CCTCGGATTC CATTGCCCAG CTATCTGTCA CTTCATCAAA 480

AGGACAGTAG AAAAGGAAGG TGGCACCTAC AAATGCCATC ATTGCGATAA AGGAAAGGCT 540

ATCGTTCAAG ATGCCTCTGC CGACAGTGGT CCCAAAGATG GACCCCCACC CACGAGGAGC 600

ATCGTGGAAA AAGAAGACGT TCCAACCACG TCTTCAAAGC AAGTGGATTG ATGTGATATC 660

TCCACTGACG TAAGGGATGA CGCACAATCC CACTATCCTT CGCAAGACCC TTCCTCTATA 720

TAAGGAAGTT CATTTCATTT GGAGAGGACA CGCTGAAATC ACCAGTCTCT CTCTACAAAT 780

CTATCTCTCT CGAGCTTTCG CGAGCTCCGA TGAAAGGCAT AAGAAAAAAC TACGAAAATT 840

CTTACACCTT ATCCTTTTTG CTTGTCTTTT TCGTCTTGAT TCTACTTCCT CCTGCCTTTT 900

CGATTAACAC TTTGTCTTCT GAAGAAGGTA CCCAATTCCG CAAAAATCCG TCAAAAATGG 960

AGTCCAAAAC AGGTGAAAAT CAAGATCGTC CCGTTTTATT GGGAGGTTGG GAAGATCGCG 1020

ATCCAAAGGA TGAAGAAATC CTGGAACTAT TGCCAAGCAT ATTGATGAAA GTAAATGAAC 1080

AATCAAACGA TGAATATCAT TTGATGCCGA TCAAATTACT GAAGGTTTCA TCTCAAGTTG 1140

TCGCTGGTGT GAAATACAAG ATGGATGTGC AGGTTGCTCG ATCGCAATGT AAAAAAAGTT 1200

CGAATGAAAA AGTTGATCTA ACAAAGTGCA AAAAATTAGA AGGACATCCT GAAAAGGTTA 1260

TGACTTTGGA AGTTTGGGAG AAACCATGGG AGAATTTTAT GCGCGTCGAA ATTCTGGGAA 1320

CAAAAGAAGT ATGA 1334

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "EXK-1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CGAATTCTCT AGAGGTACCG CATG 24

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "EXK-2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CGGTACCTCT AGAGAATTCG CATG 24

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1346 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "translational fusion"

(vii) IMMEDIATE SOURCE:
(B) CLONE: POV-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

AGACAGTATA CATAATTTAG AGAGAGTATT TTCAAGGTTT TAATCCAATT AAACATAATG 60

ATGTTTTGAT AGTCTTTAAA AAGTATTTTC ACGTTTTCAA GATAAGATAA TAACTTTGAA 120

TTTTTTTAAT TCTTGTGTAG GCTCACGTTG ACATAGTACT TCCAAAGATT TTACACATCG 180

ACAACATAAA AAAAAACACT GGTATATATA TATATATATA TATATATATA TATATAGATG 240

TTTTTAATAT TGTGTCCCCC ATTAAAAACT TTTCAAAATC TGCCTCTGCT TCTCTCTGAG 300

CTATATACAT TATAGCCTTC ATATGTTGGT TTACGATAAA TCCGTCCAAC CGTATGTTTT 360

AAACATAATG TCTCTTCTTC ACTCATGTCA ATTTCATAAG TTGGCTAACA ATTAACCTGA 420

AAAATGTACG TATCATAAAA ATGCTATAAA CGTGCACGAG TAGAACAAGT CTTTCGTCTA 480

ATAATAAACC GCTAGTTTCT CAAAATTAAA TTAGCCTAGT AATTCCTTGA TAATTGGCCA 540

AACAATCTAA AAAACGAGAC GTTGAGAGAA AAATGGGTTA AACATATCTC CATTAAGGGC 600

ACTATATAAA GCAGCAGAGG CATAGCTAAA CTCTCATAAA ACAAAACAAA TAACAATAAA 660

AAACAAATAA AAAATAAATA AATAATGGGT TCATATTTAG GAATTTATAC AATTTTGGTT 720

CTATGTTTGC TGGGATATTC AGCCAATGCT GAGGTGTTCA CCGTTGGTGG TCCTCCAGGT 780

TCTGATATTA CTGCGGTTAG TATCTATACT ATTTTCTAAT TATTTCATCC AGAGAAATCT 840

ATAACTGTTT TTTTACTTTT TTTTTGGCTA ACGTACTCGT TGGGGTTTTG TTGAAGGCTC 900

TTCTTAAAGC GTTCACATCA GCATGCGAAT TCTCTAGAGG TACCCAATTC CGCAAAAATC 960

CGTCAAAAAT GGAGTCCAAA ACAGGTGAAA ATCAAGATCG TCCCGTTTTA TTGGGAGGTT 1020

GGGAAGATCG CGATCCAAAG GATGAAGAAA TCCTGGAACT ATTGCCAAGC ATATTGATGA 1080

AAGTAAATGA ACAATCAAAC GATGAATATC ATTTGATGCC GATCAAATTA CTGAAGGTTT 1140

CATCTCAAGT TGTCGCTGGT GTGAAATACA AGATGGATGT GCAGGTTGCT CGATCGCAAT 1200

GTAAAAAAAG TTCGAATGAA AAAGTTGATC TAACAAAGTG CAAAAAATTA GAAGGACATC 1260

CTGAAAAGGT TATGACTTTG GAAGTTTGGG AGAAACCATG GGAGAATTTT ATGCGCGTCG 1320

AAATTCTGGG AACAAAAGAA GTATGA 1346

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer GUS sense-1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

GGAATTCACC GCGTCTTTGA TCGC 24

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "reverse primer NOS#2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GCGCGCGATA ATTTATCC 18

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer NPTII-121"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

GGGCGCCCGG TTCTTTTT 18

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer NPTII-B"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

CAGCAATATC ACGGGTAGCC AACGC 25

INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/CA 99/00237

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C12N15/62 C07K17/02 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 21029 A (UNIV TECHNOLOGIES INT) 11 July 1996 (1996-07-11) page 7, line 11 - line 18 ---	1-15, 19-26,42
X	US 5 275 819 A (AMER MOH S ET AL) 4 January 1994 (1994-01-04) column 7, line 50 - line 59 ---	16-18
X	WORRALL D ET AL: "PREMATURE DISSOLUTION OF THE MICROSPOROCYTE CALLOSE WALL CAUSES MALE STERILITY IN TRANSGENIC TOBACCO" PLANT CELL, vol. 4, 1 July 1992 (1992-07-01), pages 759-771, XP002035924 ISSN: 1040-4651 the whole document --- -/--	1-8, 11-13, 17-22

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 August 1999	03/09/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Maddox, A

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int. tional Application No
PCT/CA 99/00237

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 21913 A (PENN STATE RES FOUND) 17 August 1995 (1995-08-17) example 3	1,2,4, 6-8, 11-13, 19-22, 27-37
X	EP 0 436 467 A (CIBA GEIGY AG) 10 July 1991 (1991-07-10) the whole document	1,2,4, 6-8, 11-13, 19-22, 27-37
X	WO 94 09139 A (UNIV GUELPH ;PIONEER HI BRED INT (US)) 28 April 1994 (1994-04-28) the whole document	1,2,4, 6-8, 11-13, 19-22, 27-37
X	WO 93 18149 A (PIONEER HI BRED INT) 16 September 1993 (1993-09-16) the whole document	1,2,4, 6-8, 11-13, 19-22, 27-37
X	EP 0 343 947 A (LUBRIZOL GENETICS INC) 29 November 1989 (1989-11-29) page 4, line 24 - line 31	1,2,4, 6-8, 11-13, 19-22, 27-37
X	EP 0 222 526 A (LUBRIZOL GENETICS INC) 20 May 1987 (1987-05-20) page 7, paragraph 2 - page 8, paragraph 2	1,2,4, 6-8, 11-13, 19-22, 27-37
X	TORIYAMA K ET AL: "TRANSFORMATION OF BRASSICA OLERACEA WITH AN S-LOCUS GENE FROM B CAMPESTRIS CHANGES THE SELF-INCOMPATIBILITY PHENOTYPE" THEORETICAL AND APPLIED GENETICS, vol. 81, no. 6, 1 January 1991 (1991-01-01), pages 769-776, XP002069999 ISSN: 0040-5752 the whole document	28,30-37

-/--

INTERNATIONAL SEARCH REPORT

In. ational Application No
PCT/CA 99/00237

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	STAHL R J ET AL: "THE SELF-INCOMPATIBILITY PHENOTYPE IN BRASSICA IS ALTERED BY THE TRANSFORMATION OF A MUTANT S LOCUS RECEPTOR KINASE" PLANT CELL, vol. 10, 1 February 1998 (1998-02-01), pages 209-218, XP002070000 ISSN: 1040-4651 the whole document ---	28,30-37
A	MURPHY, D.J., ET AL.: "Biosynthesis, targeting and processing of oleosin-like proteins, which are major pollen coat components in Brassica napus" THE PLANT JOURNAL, vol. 13, no. 1, January 1998 (1998-01), page 1-16 XP002113174 the whole document ---	1-42
A	EP 0 270 248 A (ICI PLC) 8 June 1988 (1988-06-08) the whole document -----	1-42

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

In ational Application No
PCT/CA 99/00237

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9621029 A	11-07-1996	US 5650554 A	22-07-1997
		AU 4295096 A	24-07-1996
		BR 9600006 A	21-01-1998
		CA 2208751 A	11-07-1996
		EP 0871749 A	21-10-1998
		ZA 9510999 A	13-07-1996
US 5275819 A	04-01-1994	CA 2102211 A	03-11-1992
		US 5013552 A	07-05-1991
		EP 0583238 A	23-02-1994
		WO 9219229 A	12-11-1991
WO 9521913 A	17-08-1995	US 5585543 A	17-12-1996
		AU 1746095 A	29-08-1995
EP 0436467 A	10-07-1991	AU 642454 B	21-10-1993
		AU 6855490 A	11-07-1991
		CA 2033247 A	30-06-1991
		EP 0825262 A	25-02-1998
WO 9409139 A	28-04-1994	AU 5298593 A	09-05-1994
		US 5821094 A	13-10-1998
WO 9318149 A	16-09-1993	EP 0629241 A	21-12-1994
		PT 101209 A	31-03-1994
		US 5821094 A	13-10-1998
EP 0343947 A	29-11-1989	US 5053331 A	01-10-1991
		JP 2079979 A	20-03-1990
EP 0222526 A	20-05-1987	JP 62175188 A	31-07-1987
		US 5037959 A	06-08-1991
		US 5053331 A	01-10-1991
EP 0270248 A	08-06-1988	AT 102992 T	15-04-1994
		AU 614727 B	12-09-1991
		AU 8110487 A	12-05-1988
		DE 3789358 D	21-04-1994
		DE 3789358 T	23-06-1994
		ES 2062986 T	01-01-1995
		JP 63141591 A	14-06-1988